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Survey of aflatoxin in dairy cow feed and raw milk in China

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ABSTRACT

In August 2010, 200 feed samples for dairy cow and 200 milk samples were collected from ten major milk-producing provinces in China. The feed samples were analysed for Aflatoxin (AF) B1, B2, G1 and G2, using the HPLC method. AFM1 in the milk samples was determined using the ELISA method. AFB1 and AFB2, but not AFG1 and AFG2 were detected in the feed samples. In the feeds, 42% of the samples contained AFB1 in the range of 0.05–3.53 μg/kg, and 36% of the samples were detected positive for AFB2, with the content ranging from 0.03 μg/kg to 0.84 μg/kg. The content of AFB1 was significantly ($P < 0.05$) higher than that of AFB2 in the feeds, but it was still below the legal limits of 5 μg/kg (in EU) and 10 μg/kg (in China), respectively. The total content of AFs was below the U.S. legal limit of 20 μg/kg. For the milk samples, 32.5% were detected positive for AFM1, in the range of 5.2–59.6 ng/L, far below the legal limit of 500 ng/L in China and the US. However, three samples contained AFM1 at the levels of exceeding 50 ng/L of the EU legal limit. Furthermore, there was no significant ($P > 0.05$) difference between the north and the south of China in the AF contents in both the dairy cow feed samples and the milk samples.

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1. Introduction

Aflatoxins (AFs) are toxic secondary metabolites produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. They could cause acute liver damage, liver cirrhosis and hepatocellular carcinoma, along with immunosuppressive effects (Alborzi, pourabbas, Rashidi, & Astaneh, 2006). Eighteen AFs have been identified up to now, but only four of B1, B2, G1 and G2 are most concern (Decastelli et al., 2007). Among them, AFB1 is the most toxic and has been designated as group 1 carcinogenic compound by the International Agency for Research on Cancer (IARC) (IARC, 1993).

AFB1 is a contaminant found in feeds that have been improperly dried. Dairy cows consuming AFB1-contaminated feeds accumulate a hydroxylated metabolite of AFB1 known as AFM1 in the milk (Diaz & Espitia, 2006). AFM1 could be detected in milk within 12–24 h of an animal consuming feed containing AFB1, and decreased to an undetectable level after 72 h (Battacone et al., 2003; Martins & Martins, 2000). AFB1 consumed in feed could be transformed to AFM1 in milk, and the transformation varies from day to day and from one milking to another one. Transformation rates of AFB1 in ingested feed to AFM1 in cow's milk varied in different reports, from 0.032% (Battacone et al., 2003), 1–3% (Sassahara, Pontes, & Yanak, 2005) to 6% (Pitet, 1998).

AFM1 has lower toxicity than AFB1, and it has been classified as group 2B carcinogenic compound by the IARC (IARC, 1993). It is assumed that neither storage nor processing, such as pasteurisation, autoclaving or other methods, could destroy the AFM1 toxin (Tajkarimi et al., 2008). The level of AFM1 in raw milk is a concern due to its mutagenic, carcinogenic, and teratogenic effects (Sassahara et al., 2005). To reduce exposure to AFM1, the legal limit of AFM1 in raw milk has been established by the regulatory authorities. Japan and the U.S. have a legal limit of 500 ng/L, whereas it is 50 ng/L in the European Union (EU). These standards have been adopted by many other countries (Commission Regulation [EC], 2006; FAO, 2004). Moreover, the level of AFB1 contamination in feeds determines the level of AFM1 in raw milk. Thus, a legal limit for AFB1 in dairy cow feeds has also been established. The legal limit is set at 10 μg/kg in Japan and Korea, and 5 μg/kg in the EU. The U.S. and Canada have set the legal limit of 20 μg/kg for total AFs (AFB1+AFB2+AFG1+AFG2) in dairy cow feeds (EC, 2006; FAO, 2004).

China has established the legal limits of 500 ng/L for AFM1 in milk and 10 μg/kg for AFB1 in dairy cow feeds, respectively. However, there

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are no report on the level of AFM1 in raw milk and the levels of AFs in dairy cow feeds in China. In the present study, HPLC analysis of AFB1, AFB2, AFG1 and AFG2 in dairy cow feeds and ELISA analysis of AMF1 in raw milk were performed for 200 feed samples and 200 milk samples collected from ten provinces in China.

2. Material and methods

2.1. Sampling

During August 2010, 200 samples of raw milk and 200 samples of dairy cow feeds were collected from 200 dairy farms in ten provinces in China, including Heilongjiang, Inner Mongolia, Hebei, Shandong, Ningxia, Shanxi, Beijing, Tianjin, Shanghai and Guangdong. The temperature and humidity that indicated by rainfall in these regions (NCC/CMA, 2010) are shown in Table 1. The dairy cow feed samples were taken from the feed fed to the cows from which the milk samples were obtained. The feed samples were collected on the same day as the milk sampling.

The raw milk was collected directly from milk-holding tanks at milk stations on the dairy farms. After stirring the milk-holding tank, each 200 mL of milk was taken from the upper third, the middle third and the lower third of the tank, respectively. The total 600 mL of milk from each tank was mixed, from which a 100 mL sample was taken and stored at −20 °C until the analyses were performed.

Feed samples were collected from sacks, which were selected randomly, with each 2 kg of feed taken from the upper third, the middle third and the lower third of each sack. The total 6 kg of feed was mixed, from which 0.5 kg of the sample was collected and heated at 65 °C to remove moisture. It was then stored at room temperature until analysed.

2.2. Feed sample analysis using the HPLC method

2.2.1. Extraction

The feed samples were ground into fine particle. Ground feed (25.0 g) was mixed with 125 mL of 70% (v/v) methanol (chromatographic grade, Merck KGaA, Darmstadt, Germany) and 5.0 g of NaCl in a 250 mL triangular flask, and then sonicated for 30 min. The mixture was then filtered with quantitative filter paper, and the filtrate was diluted twice with Milli Q water. The diluted filtrate was retained for purification.

2.2.2. Purification

Immunoaffinity columns (Aflaprep, R-Biopharm Rhone Ltd, Scotland, U.K.) were used to purify AFB1, AFB2, AFG1 and AFG2. Fifteen millilitres of filtrate were placed in a syringe, which was connected to the immunoaffinity columns. After the filtrate flowed through the gel, AFB1, AFB2, AFG1 and AFG2, which might be present in the samples, would be captured by the antibodies on the gel. Afterwards, the column was washed with 20 mL of Milli Q water to remove extraneous nonspecific substances. The bound AFB1, AFB2, AFG1 and AFG2 were eluted from the column with 1 mL of methanol. The elution was then diluted with 1 mL of Milli Q water.

Table 1
Temperature and humidity in August, 2010 in ten provinces, China.

Province	Temperature (°C)	Rainfall (mm/month)
North:		
Heilongjiang	20.1	125.2
Inner Mongolia	20.3	53.8
Beijing	25.8	145.0
Tianjin	26.0	147.6
Ningxia	21.2	33.6
Hebei	23.7	177.6
Shanxi	22.0	146.3
Shandong	25.0	273.3
Average	23.0	137.8
South:		
Shanghai	30.8	203.8
Guangdong	28.6	146.3
Average	29.7	175.1

Humidity is presented by rainfall in each province.

2.2.3. Quantitation

Determination of the AFB1, AFB2, AFG1 and AFG2 contents was carried out with a HPLC system (Waters, Milford, MA, USA) equipped with a 2695 separation module, a 2475 fluorescence detector and Empower professional software (Empower Software Solutions, Inc., Orlando, FL, USA). The separation of AFB1, AFB2, AFG1 and AFG2 was achieved with a mycotoxin analysis column (4.6 mm × 250 mm, Mycotox, Pickering laboratories, Mountain view, CA, USA). Methanol-acetonitrile-water (22–22–56, V–V–V) was used as the mobile phase at the flow rate of 1.0 mL/min. A post-column derivatising system (Vector pcx, Pickering laboratories, Mountain view, CA, USA) was employed after the separation of AFB1, AFB2, AFG1 and AFG2, and iodine topical (chromatographic grade, Merck KGaA, Darmstadt, Germany) solution (0.01%, W:V) was used as a derivatising reagent with a flow rate of 0.4 mL/min. AFB1, AFB2, AFG1 and AFG2 were monitored at an excitation wavelength of 365 nm and an emission wavelength of 430 nm and quantified by co-chromatography with authentic AFB1, AFB2, AFG1 and AFG2 standards (Sigma Aldrich, Inc., St Louis, MO, USA).

2.3. Milk sample analysis using the ELISA method

The quantitative analysis of AFM1 in the milk samples was determined using a RIDASCREEN® Aflatoxin M1 test kit of competitive enzyme immunoassay (R1111, R-Biopharm AG, Darmstadt, Germany). All reagents required for the enzyme immunoassay, including standard of AFM1 (0, 5, 10, 20, 40 and 80 ng/L), were included in the test kit.

2.3.1. Sample preparation

Eight millilitres of milk was defatted by centrifugation at 3500 × *g* and 10 °C for 10 min. The upper cream layer was removed, and the defatted supernatant was subjected to the ELISA test for AFM1.

2.3.2. Test procedure

One-hundred millilitres of the standard solutions or the defatted samples were added in wells and incubated for 30 min at room temperature (20–25 °C) in the dark. The liquid was then sucked out of the wells, and all the wells were filled with 250 mL of washing buffer before sucking out the liquid again. The washing procedure was repeated twice. Next, 100 μL of the diluted enzyme conjugate was added in the wells and incubated for 15 min at room temperature in the dark. This washing procedure was repeated two more times. Then, 100 μL of chromogen was added to each well and incubated for 15 min at room temperature in the dark. Lastly, 100 μL of the stop solution was added to each well, and the absorbance was measured at 450 nm by a microplate reader (Infinite 200, Tecan Austria GmbH, Groedig, Austria).

2.3.3. Result calculation

The content of AFM1 in the samples was calculated using specialised RIDA®SOFT Win software (Z9999, R-Biopharm AG, Darmstadt, Germany). The detection limit of the present method is 5 ng/L for milk.

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