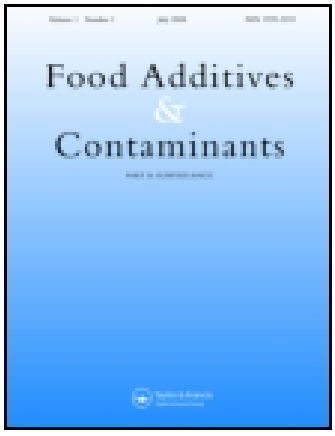

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Aflatoxin M_1 in raw cow and buffalo milk in Shush city of Iran

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Aflatoxin M_1 (AFM_1) contamination is evaluated in 120 samples of raw milk from cow and buffalo (60 each), collected randomly in the Shush (southwest Iran). Enzyme-linked immunoabsorbent assay (ELISA) was applied to analyse AFM_1 in the samples. AFM_1 was detected in 44 (69%) raw cow milk samples with a mean of 55 ng/l at a range of 3.6–419 ng/l) and in 46 (79%) raw buffalo milk samples with a mean of 116 ng/l at a range of 13–423 ng/l. In all samples, the AFM_1 concentration was lower than the Iranian national standard and FDA limit of 500 ng/l. According to the European Union and Codex Alimentarius Commission, 18 (28%) and 32 (52%) of cow and buffalo raw milk samples are above the 50 ng/l limit, respectively. Results showed that AFM_1 contamination of raw milk could pose a problem for public health, since all age groups, including infants and children, consume this product.

Keywords: Aflatoxin M_1; raw milk; cow; buffalo; ELISA; Iran

Introduction

Aflatoxins (AFs) are the best known and most intensively researched mycotoxins worldwide, which are produced by different *Aspergillus* (A) species, like *A. flavus*, *A. parasiticus*, *A. bombycis*, *A. ochraceoroseus*, *A. nomius* and *A. pseudotamarii*. It contaminates agricultural commodities and thus food and feed, particularly in critical temperature and humidity conditions before or during harvest, or because of inappropriate storage (Lee et al. 2009; Firdous et al. 2012). The main types of AFs are B_1, B_2, G_1 and G_2 which are currently produced in plant product. Biotransformed AFs may occur in milk and dairy products, such as AFM_1 and AFM_2 (Tavakoli et al. 2012, 2013). AFM_1 is the monohydroxylated metabolite of AFB_1 formed in liver by means of microsomial cytochrome P450- associated enzymes and excreted through body fluids such as milk, urine, faeces and blood (Ardic et al. 2009; Ghanem & Orfi 2009).

There is a linear relationship between the amount of AFM_1 in milk and AFB_1 in the feed which is consumed by animals (Bakirci 2001; Abbes et al. 2012). Monitoring studies reported that approximately 0.3–6.2% of AFB_1 ingested by livestock is transformed to AFM_1 in milk. However, transmission rate varies from animal to animal, day to day and one milking process to the next. AFM_1 could be detected in milk 12–24 h after the first ingestion of AFB_1. When the intake of AFB_1 is stopped, the AFM_1 concentration in the milk decreases to an undetectable level after 72 hours (Van Egmond 1989; Ardic 2009; Fallah 2010a). The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) included AFB_1 as primary and AFM_1 as secondary group of carcinogenic compounds (Tavakoli et al. 2012). Several studies showed that AFM_1 is relatively stable to heat and high temperature with pasteurisation, sterilisation (UHT techniques), autoclaving, freezing, fermenting, preparation and cold-storage of various dairy products and also the level of AFM_1 does not change in contaminated milk (Bakirci 2001; Abbes et al. 2012; Tavakoli et al. 2013). Most of the developed countries have set or proposed legal regulations for AFM_1 levels in milk and dairy products to reduce this hazard. These regulations vary from one country to another country and are dependent on economic considerations (Fallah 2010b; Rahimi et al. 2010).

The Khuzestan province of Iran is a major source for abundant milk production, obtained from cow, buffalo, goat, sheep and camel. Therefore, this study was carried out with the objective of investigating the presence of AFM_1 in raw milk from cow and buffalo in southwest Iran.

Materials and methods

Samples

A total of 120 samples of raw milk, cow (n = 60) and buffalo (n = 60), were taken randomly from different parts of Shush in Khuzestan province of Iran during 6 months (February to July 2012). These months were divided into

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three seasons: winter, spring and summer. All samples were transported to the laboratory in an icebox at 2–4°C and kept at –20°C until analysis.

Sample preparation

Raw milk samples were centrifuged at 2000 *g* for 5 min. After centrifugation, the upper fat layer was removed by aspirating with a Pasteur pipette and used for AFM_1 analysis.

Analysis

Quantitative analysis of AFM_1 in the raw milk samples was performed by competitive enzyme-linked immunoabsorbent assay (ELISA) using an AFM_1 kit (Euro Clone, EuroClone S.p.A., Pero (Milano) Italy). Exactly 200 µl of the AFM_1 standard solutions and test samples in duplicate were added to the wells of micro-titer plates pre-coated with antibodies for AFM_1 and incubated for 30 min at room temperature (20–25°C) in the dark. The liquid was poured out and the wells were filled with approximately 300 µl washing buffer and the liquid was poured out again. This washing step was repeated twice. In the next stage, 200 µl of enzyme conjugate were added to occupy the remaining free binding sites and in washing step 250 µl of washing buffer washed any unbound enzyme conjugates. Then, 50 µl of enzyme substrate and 50 µl of chromogen were added to the wells and incubated for 10 min at room temperature in the dark. The reaction was stopped by adding 50 µl stop solution to each well and absorbance was measured at 450 nm in a spectrophotometer ELISA plate reader. Results were expressed as the means of two analyses. The absorbance obtained for standards and samples were divided by the absorbance of the first standard (the zero standard) and multiplied by 100. Therefore, the zero standards are considered 100% and the absorbance values are expressed in percentage.

Statistical analysis

Data were analysed by Statistics 19 SPSS IBM software (http://ibm-spss-statistics.soft32.com/old-version/69/19/). To evaluate differences in the means between months and two groups of samples, *t*-test and ANOVA for independent samples were applied. Differences between values were considered significant at $P \leq 0.05$.

Results

The standard curve was linear from 50 to 100 ng AFM_1/l. The correlation coefficient R^2 was 0.9998. The limit of detection was defined as the minimum level at which the analyte could be detected and was determined to be 3 ng/l. The incidence and levels of AFM_1 in raw cow and buffalo milk samples are presented in Tables 1 and 2, respectively. The concentration of AFM_1 in all samples was lower than the Institute of Standards and Industrial Research of Iran and FDA limit of 500 ng/l (US FDA 1996; ISIRI 2005). But, 28% (18/60) of cow milk and 52% (32/60) of buffalo milk samples contained higher levels than the maximum limit of 50 ng/l of the European Union and Codex Alimentarius Commission (Codex Alimentarius Commission 2001; European Commission 2006). Regarding seasonal effect influences, mean concentration of AFM_1 in the buffalo samples collected in the three seasons was significantly ($P < 0.05$) higher than in cow samples.

Discussion

AFM_1 contamination of milk and milk products is the result of feeding cows' material containing AFB_1. The concentration of AFB_1 in animal feed is influenced by feed type, time and method of harvesting, temperature and moisture content. *A. flavus* and *A. parasiticus* can easily grow in feed having a moisture content between 13–18% and an environmental humidity between 50–60% (Bakirci 2001; Ardic 2009). Our findings revealed a high incidence of AFM_1 in raw milk samples. Also, statistical analysis of the data shows that the percentage of AFM_1 contamination in buffalo milk is more than in cow milk, which was not similar to a study conducted by Rahimi et al. (2010). In another study, Bilandzic et al. (2010) reported less AFM_1 (1.6%) at detectable levels in raw milk samples; but in a recent study, Davoudi et al.

Table 1. Distribution by season of aflatoxin M_1 (ng/l) in 60 raw cow milk samples.

Season	Tested	Negative[a]	Positive[a]	Minimum	Maximum	Mean ± SE[b]	Exceeding regulation,[c] *n* (%)
Winter	10	6 (60%)	4 (40%)	4.5	14.1	6.1[a] ± 2.7	0 (0)
Spring	30	10 (33%)	20 (67%)	3.6	100.9	32.6[a] ± 8.4	4 (13)
Summer	20	0 (0%)	20 (100%)	23.2	419.5	114.9[b] ± 36.3	14 (70)
Total	60	16 (31%)	44 (69%)	3.6	419.5	55.5	18 (28)

Notes: [a]Values in parentheses indicate % negative and positive samples.
[b]Mean ± SE with different letters is significantly different.
[c]The EU limit for AFM_1 is 50 ng/l for raw milk.

Table 2. Distribution by season of aflatoxin M_1 (ng/l) in 60 raw buffalo milk samples.

Season	Tested	Negative[a]	Positive[a]	Minimum	Maximum	Mean ± SE[b]	Exceeding regulation,[c] *n* (%)
Winter	10	2 (20%)	8 (80%)	19.1	209.5	93.2[a] ± 47.1	4 (40)
Spring	30	10 (33%)	20 (67%)	19.5	422.7	109.1[a] ± 40.5	14 (47)
Summer	20	2 (10%)	18 (90%)	12.7	367.3	138.4[a] ± 40.9	14 (70)
Total	60	14 (21%)	46 (79%)	12.7	422.7	116.2 ± 25.1	32 (52)

Notes: [a]Values in parentheses indicate % negative and positive samples.
[b]Mean ± SE with different letters is significantly different.
[c]The EU limit for AFM_1 is 50 ng/l for raw milk.

Table 3. Occurrence and levels of aflatoxin M_1 in raw milks reported in previous studies.

Country	Samples	Positive (%)	Range (ng/l)	Method	References
India	12	33	28–164	ELISA	Rastogi et al. (2004)
Iran	111	76.6	15–280	TLC	Kamkar (2005)
Iran	98	100	3–329	HPLC	Tajkarimi et al. (2007)
Turkey	90	88.9	17–232	ELISA	Ardic (2009)
Turkey	45	91.1	75	ELISA	Kart et al. (2009)
Syrian	108	83.3	6–690	ELISA	Ghanem and Orfi (2009)
Iran	311	42.1	50	ELISA	Rahimi et al. (2010)
Croatia	61	1.6	0.6–58.7	ELISA	Bilandzic et al. (2010)
Iran	100	100	30–630	TLC	Davoudi et al. (2011)

(2011) determined AFM_1 in 100% of the investigated raw milk samples. In previous surveys conducted in Iran by TLC methods, Kamkar (2005) reported that 77% (85 of 111) raw milk samples were contaminated with AFM_1 in Sarab and 40% had levels above the European limit. A survey from Morocco (El Marnissi et al. 2012) reported AFM_1 in raw milk in 13 out of 48 samples (27%), ranging 10–100 ng/kg and within the positive samples 4 (~8% of the total) were above the EU limit. A study on raw milk samples in Lebanon showed that 28 (74%) of 38 raw milk samples contained AFM_1 and 61% of the positive samples were higher than the EU limit (Assem et al. 2011). Similar results were documented from Tunisia by Abbes et al. (2012), who found by an ELISA method that 61% of 112 raw milk samples included AFM_1. An earlier study by Lee et al. (2009) from South Korea reported 48 (48%) of 100 raw milk samples contained AFM_1. In Punjab, Pakistan, Hussain et al. (2008) analysed 480 raw milk samples, 360 of buffalo and 120 of cow. Percentages of AFM_1 contamination in buffalo and cow milk were 42.5 and 52.5%, respectively. Several studies by other researchers are shown in Table 3.

Conclusions

AFM_1 is determined in a high percentage of milk samples, which can justify to perform a monitoring programme for the total milk chain, as especially infants and children consume these products. Control could start from the farm, by regular control of AFB_1 contamination of animal feed and control of its storage conditions. The use of a food control system, like the HACCP system in the food industries, is suggested as an efficient means of limiting mycotoxin contamination, especially for AF in Iranian food.

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