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Occurrence of Aflatoxin M1 in raw and pasteurized milk produced in west region of Iran (during summer and winter)

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Abstract

In the present study, 144 milk samples (102 raw milk samples and 42 pasteurized milk samples) in west region of Iran were examined for aflatoxin M1 by ELISA. AFM1 was found in 47.91% of the samples by average concentration of 39.45 ± 18.40 ng/l. The highest mean concentration of aflatoxin M1 was registered in traditional dairy farm samples (43.9 ± 9.5 ng/l). The concentration of AFM1 in 21.5% of raw cow milk and 11.9% pasteurized milks were higher than maximum tolerance limit accepted by European union/Codex Alimentarius Commission (50 ng/l). The level of contamination in winter was significantly ($P < 0.05$) higher than in summer.

Introduction

Milk is a valuable source of nutrients and it's widely used in many countries even as a complete nutritional meal is also considered. However, milk can be considered as a source of toxic compounds such as mycotoxins (Ghazani, 2009; Fallah, 2010). Aflatoxin M1 (AFM1), known as milk toxin, is the principle hydroxylated product of AFB1, metabolized by cytochrome P450 associated enzymes in liver and appear in milk, feces and urine of lactating animals following consumption of the AFB1 contaminated ration (Aycicek *et al*., 2005; Fallah *et al*., 2009). It could be detected in milk within 12 h after the first ingestion of AFB1. Following the withdrawal of contaminated source, AFM1 concentration in the milk decreased to an undetectable level within 72 h (Rahimi and Karim, 2008). Due to the widespread consumption of milk and dairy products, presence of AFM1 in these products has become a worldwide concern (Fallah *et al.*, 2009).

Several researchers have been reported of potential hazardous human exposure to AFM1 through milk and milk product (Sassahara *et al*., 2005; Unusan, 2006; Oveisi *et al*., 2007). So many countries to reduce this risk proposed legal regulations for AFM1 levels in milk and dairy products. In addition, these regulations vary in different countries due to economic considerations (Stoloff *et al*., 1991). A maximum acceptable limit of 50 ng/l for AFM1 in milk has established by the European Commission (Anonymous, 2000). However, acceptable limit level of AFM1 set by the Institute of Standards and Industrial Research of Iran was 500 ng/l (ISIRI, 2005), which is the same as US Food and Drug Administration (FDA, 2000) accepted level. The purposes of this study, therefore, were to ascertain: Assessment of AFM1 contamination differences between milks from traditional, industrial dairy farms and pasteurized milk; difference between spring and summer levels of AFM1 contamination and compare the results with the legal regulations for AFM1 legislated by EC, US FDA, and ISIRI.

Materials and Methods

Sample preparation

A total of 144 samples of raw and commercial pasteurized milk were collected from traditional, industrial dairy farms and supermarkets located in Ilam, as described in Table 1. The samples collected included 54 traditional dairy farms (from milk collecting centers), 48 industrial dairy farms (from the raw milk tankers arriving directly from industrial dairy farms) and 42 pasteurized milks (different brands were purchased randomly from large supermarkets and retail shops). Our sampling scheme was based on the relative volumes of milk for better precision, and we tried to get each season's samples from the same farm and/or milk collecting center. We collected the samples during February 2011 (winter indicator) and August 2012 (summer indicator). All of the milk samples transported at 2–4

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°C in an icebox and analyzed by direct competitive enzyme-linked immunosorbent assay (ELISA) for presence of AFM1.

Method for analysis of AF M1

The quantitative analysis of AFM1 in the milk samples was performed by competitive enzyme immunoassay using Euroclone_ Aflatoxin M1 Elisa kit (Quantative EuroClone Aflatoxin M1, Cod. EEM005096. LOT. AM11110V).

Preparation of milk samples

Preparation of milk samples was conducted according to the instructions of kit. Milk samples were chilled to 10°C and then centrifuged at 2000*g* for 5 min. The upper creamy layer was completely removed by aspirating through a Pasteur pipette and from the lower phase (defatted supernatant) 200 µl was directly used per well in the test.

ELISA test procedure

ELISA test procedure was conducted according to the instructions of kit. 200 µl of standard solutions (were provided in 0, 5, 10, 25, 50 and 100 ng/l concentrations) and prepared samples were added into separate microplate wells and incubated for 30 min at room temperature (20–25°C) in the dark. The liquid was then poured out and the wells were washed with washing buffer (250 µl) thrice. In the next stage, 200 µl of the diluted enzyme conjugate was added to the wells, mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Again, the wells were washed thrice with washing buffer. Afterwards, 200 µl of substrate/ chromogen was added, mixed gently and incubated in the dark at room temperature for 15 min. Finally, 50 µl of the stop reagent was added into the wells and the absorbance was measured at k = 450 nm in ELISA plate reader against air blank within 15 min. According to the Euroclone AFM1kit guidelines, the lower detection limit is 5 ng/l for milk.

Statistical analysis

The statistical methods used in this study were based on normal confidence intervals and analysis of variance (ANOVA). The levels were considered significantly different at $P < 0.05$.

Results

The results of the analyses of AFM1 level (ng/l) in raw and pasteurized cow milk are shown in Table2. The presence of AFM1 was observed in 43.7% of all samples. The overall mean level of AFM1 in the samples was 39.4 ± 5.1ng/l (Table1). However, none of the samples was higher than the maximum tolerance level of AFM1 in liquid milk regarding Iranian national standard, and FDA standard (500 ng/l), (FDA, 2000), (ISIRI, 2005). But, 27 (18.7%) of samples was higher than maximum tolerance limit accepted by European Union (EU) and Codex Alimentarious Commission (50 ng/l), (Anonymous, 2000).

The mean concentration of AFM1 in various type of analyzed milk was significantly ($P < 0.05$) differences (Table1), the highest mean concentration of AFM1 was registered in traditional dairy farm samples (43.9 ± 9.5 ng/l). (34.2 ± 2.1ng/l). Also the mean concentration AFM1 in traditional dairy farm milk samples was significantly ($P < 0.05$) higher than industrial dairy farm milk samples (34.2 ± 2.1ng/l). Moreover, based on our results, the mean concentration AFM1 in pasteurized milk (36.0 ± 4.6) was remarkable.

The incidence rates of AFM1 in raw milks was 66.6% and 37.2% in winter and summer season respectively, also 42.8% and 33.3% was for pasteurized milk samples. Also the mean concentration of AFM1 in all milk samples in winter was significantly ($P < 0.05$) higher than those obtained in summer (Table2).

Discussion

Raw milk production in Iran is done by the industrial and traditional dairy farms, feeding in industrial dairy farms are controlled, also used as a nutrient supplement, in this type of farming cattle usually will be in places where it is easy to provide veterinary services, hygienic and marketing service. Traditional dairy farming is most common system in Iran and cattle maintained in village areas, in this type of system cow feed is on farms and ranches. Milk is often consumed by household and the excess is transferred to the milk collection centers (Tajkarimi *et al.*, 2008). Factors such as season, time consumption and improper handling of food can be involved in the presence of AFM1 in milk. In addition, the amounts of AFM1 in the rainy season is greater than the dry season (Jonsyn-Ellis, 2000). In this study February and August, which we choose as season indicator months for sampling (Table 2).

Sorghum silage use as a main source of energy in feeding systems of the industrial dairy farms, Sorghum has been considered as an important source of the AFB1 (Da Silva *et al*., 2004). There is a linear relationship between AFB1 in dietary intake of animals and levels of AFM1in milk (Dragacci *et al*., 1995; Wood, 1991). Some internationally published

Table 1. Levels of AFM1 in various types of milk samples

Type of milk sample		Sample size(n)	Positive samples n (%)	Concentration (ng/l)				
				Total samples (mean ± SD)	Positive samples		Exceed legal limit n (%)	
					mean ± SD	Min–Max	ISIRI and US FDA[a]	EC[b]
Raw milk	Traditional farms	54	34(62.98)	43.98±9.50[a]	56.39±8.50	50.03-85.24	0	31(57.40)
	Industrial farms	48	19(39.60)	34.21±2.11[b]	53.15±3.89	50.19-60.71	0	8(16.50)
Pasteurized milk		42	10(23.80)	36.06±4.68[c]	54.39±4.68	50.03-62.66	0	6(14.28)
Total		144	63(43.75)	39.45±5.12		10.03-85.24	0	45(31.25%)

a Institute of Standards and Industrial Research of Iran (ISIRI) and US Food and Drug Administration (US FDA) limits for AFM1 in milk are 500 ng/l.
b European Commission (EC) limit for AFM1 in milk is 50 ng/l.
Means ± SEM in the column with different letters are significantly different (P < 0.05).

Table 2. Levels of AFM1 all milk samples: Comparison between samples obtained in winter and summer

Type of milk samples		Summer		Winter	
		Sample size	Concentration (mean ± SD) (ng/l)	Sample size	Concentration (mean ± SD) (ng/l)
Raw milk	Traditional	27	42.76±2.25[a]	27	44.64±1.81[b]
	Industrial	24	22.93±1.75[a]	24	40.79±1.05[b]
Pasteurized milk		21	26.67±2.01[a]	21	43.38±1.14[b]
Total		72	33.09±2.19[a]	72	43.30±1.49[b]

Means ± SEM in the same row with different letters are significantly different (P < 0.05).

data are available on the occurrence of AFM1 in raw and commercial milk in Iran. Evaluation of the AFM1 contamination in pasteurized milk samples (Six hundred and twenty four) in Shiraz city (Iran) showed that AFM1 was found in 100% of the examined milk samples. 17.8% of the samples had AFM1 greater than the aximum tolerance limit (50 ng/l) accepted by European Union (Alborzi *et al*., 2006).

In a study, 319 raw milk samples were collected from dairy farms and milk collecting centers of 15 dairy plants in 14 Iranian states in winter and summer. Samples were analyzed for aflatoxin M1 with HPLC method. Aflatoxin M1 contamination was detected in 54% of the field samples. The sample-mean concentration of aflatoxin M1 was 0.057 µg/kg. 44% of the samples had levels <0.01 µg/kg and 77% were <0.05 µg/kg. The levels of contamination in industrial and traditional dairy farms were equal, but the season had an indirect effect (Tajkarimi *et al.*, 2007). In another study, Oveisi *et al.* (2007) showed that the 78% of liquid milk samples analyzed by ELISA method were contaminated with AFM1, and AFM1 level in 33% of this milk samples was higher than the maximum tolerance limit accepted by European Union (Oveisi *et al*., 2007). Fallah *et al.* (2010) were examined AFM1 contamination dairy products (consisting pasteurized milk, yoghurt, white cheese, butter and ice cream) collected from popular markets in four large Iranian cities by thin layer chromatography (TLC) technique. The toxin was detected in 72.5% pasteurized milk samples, 66.1% yoghurt samples, 81.9% white cheese samples, 25.8% butter samples and 69.4% ice cream samples. The concentration of AFM1 in 36.2% were higher than Iranian national standard limits. Screening survey to determine the occurrence of AFM1 in 225 commercial liquid milk samples obtained from popular markets in central part of Iran indicate that AFM1 was detected in 67.1% samples, Considering the US FDA and Iranian national standard limits for AFM1 in milk (500 ng/l), 2.0% samples milk had levels above the maximum tolerance limit, However, according to European Commission limit (50 ng/l), this figure increased to 22.2% (Fallah, 2010).

Our results indicate that AFM1 was found in 59.72% and 36.11% milk samples collected in winter and summer respectively (Table2). The incidence rates of AFM1 in raw milks was 66.6% and 37.2% in winter and summer season respectively, also 42.8% and 33.3% was for pasteurized milk samples. Also the mean concentration of AFM1 in all milk samples in winter was significantly (P < 0.05) higher than those obtained in summer (Table2).

In view of the high levels of AFM1 in milk detected by us (especially in winter), and according to numerous authors, a seasonal effect influences

AFM1 occurrence. Higher incidence of AFM1 contamination during cold seasons than hot ones has been expressed by many researchers (Fallah, 2010; Tajkarimi *et al*., 2008; Ayar *et al*., 2007; Hussain and Anwar, 2008; Kamkar, 2005; Ruangwisesand, 2009). Because in winter cows fed diets containing high levels of AFB1 and a relationship between AFM1 occurrence level in milk and AFB1 content of feed was reported (Kamkar, 2005; Tajkarimi *et al.*, 2008). Out-pasturing of milking cows was the most important factor in the low levels of aflatoxin in milk in summer and spring seasons (Applebaum *et al*., 1982; Blance and Karleskind, 1981; Kamkar, 2005; Tajkarimi *et al*., 2008) and findings of these researchers also demonstrated low levels or absence of AFM1 in the summer season. Therefore it is possible to say that the results obtained in present study were parallel to the results of prior Studies.

However, according to Table1, there is a meaningful and significant effect of the farm type on the level of AFM1 contamination (Table 1). Milk samples collected from industrial farms had low contamination AFM1 (39.5%) compare on traditional dairy farms samples (62.9%), which could be related to specific feeding systems in this state. In the present study the incidence of AFM1 contamination in pasteurized milk samples was relatively high, since the toxin was detected in 38.09% of the samples. The range of contamination was between 10.0 and 62.6 ng/l, with an average value of 36.0 ± 17.4 ng/l. AFM1 was found in 38.0% pasteurized milks and 14.2% of the samples had AFM1 in excess of the maximum tolerance limit i.e. 50 ng/l (Table 1). In previous studies conducted in different cities of Iran, a high incidence of AFM1 in pasteurized milk samples at high was levels found. For example, in Babol (Gholampour *et al*., 2007), Esfahan (Rahimi *et al*., 2009), Tabriz (Ghazani, 2009) and Tehran (Oveisi *et al*., 2007), all of the examined samples contained AFM1, with mean values of 0.2, 0.1, 0.1 and 0.1 µg/l, respectively. When comparing our result with some European countries, it is higher than those found in Italy (Capei and Neri, 2002), Greece (Roussi *et al*., 2002) and Portugal (Martins and Martins, 2000). This may be because of good agricultural handling and storage practices carried out in European countries to control the risk of toxicogenic fungi contamination all along the feed supply chain; and also setting stringent regulatory limits for aflatoxins in feed and milk (Kamkar, 2005; Tajkarimi *et al*., 2008).

According to the results obtained in this study and other studies in Iran and other countries, the presence and high levels of aflatoxin in milk and other dairy products has become a serious concern for human health, In this regard, the necessary education about the health consequences of aflatoxins in food animals and food products origin from animal (particularly milk and dairy product) for dairy farmers to be given by the relevant authorities.

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