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OCCURRENCE OF AFLATOXIN M1 IN RAW MILK PRODUCED IN ARDEBIL OF IRAN

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ABSTRACT

Contamination of milk and dairy products to aflatoxin M1 is a risk for human and it can be a public heath concern. The aim of this study was to evaluate the presence of aflatoxin M1 in raw milk samples produced in Ardebil City (Iran) by ELISA (Enzyme Linked Immuno Sorbent Assay) technique. 122 samples of raw milk were collected from milk collecting centers and dairy plants in the region and aflatoxin M1 contamination was detected in all of milk samples. The mean concentration of aflatoxin M1 was 40.01ng/L and 14.75 percent of the samples had higher levels than the maximum recommended limits by ISIRI, European Community and Codex Alimentarius. With a view of the fact that milk is used by all the age groups including infants and children in the city of Ardebil, it is necessary to apply an ideal recommended limit to minimize the health hazard from aflatoxin M1 contamination in milk. Application of Good Agricultural Practices and Good Veterinary Practices by agriculture and also the Hazard Analysis and Critical Control Points (HACCP) system as a draft code of practice for preharvest and postharvest control of dairy cow's feed and in milk and dairy products processing is effective.

Key words: Aflatoxin M1; Raw milk; Enzyme linked immuno sorbent assay; Milk safety

INTRODUCTION

Aflatoxins are generally produced in animal feed by toxigenic fungi such as *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nomius*. There are fungi spores in the environment and poor storage of commodities after harvest allow the growth of fungi. Some studies have shown that various foods, peanuts and corns can be contaminated by *Aspergillus fungi* (Khanafari *et al*., 2007; Rostami *et al*., 2009). The conditions of high humidity and warm temperatures can give rise to the highest levels of aflatoxins in food (Adams and Moss, 2005). They are toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds to animals and humans (Piva *et al*., 1995; Peraica *et al*., 1999; Kocabas and Sekerel, 2003).

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Aflatoxin (AFM1) is a hydroxylated metabolite of aflatoxin B1 that may be found in milk and milk products obtained from livestock that have ingested contaminated feed (Creppy, 2002). Contamination of milk and dairy products to AFM1 can lead to increase the risk of hepatocellular carcinoma and liver cancer (Krogh, 1987; Peraica *et al*., 1999). International Agency for Research on Cancer (IARC) of WHO considers aflatoxin B1 as primary and aflatoxin M1 as secondary groups of carcinogenic compounds (Cathey *et al*., 1994; Dragacci *et al*., 1995).

The main feed sources of aflatoxins are peanut meal, maize and cottonseed meal. Many researchers reported that there was a linear relationship between the amount of aflatoxin M1 in milk and aflatoxin B1 in feed consumed by animals (Bakirci, 2001). Milk usually may

have the greatest demonstrated potential for introducing aflatoxin residues from food animal origin into the human diet (Stoloff, 1977). Many countries have established regulations to control the levels of aflatoxin B1 in feeds and to have maximum permissible levels of aflatoxin M1 in milk to reduce this hazard (Van Egmond, 1989; Sarimehmetoglo *et al*., 2004). The European Community and Codex Alimentarius Commission prescribed that the maximum level of aflatoxin M1 in milk and milk products should not exceed 50 ng/L (EC, 1992; CAC, 2001).

Some studies have been done about aflatoxin M1 contamination in raw milk in the world and their results have presented exceeded concentrations regarding the European Community and Codex Alimentarius regulatory limit (Dashti *et al*., 2009; Ghanem and Orfi, 2009; Hussain *et al*., 2008). Although, there are some studies about the contamination of aflatoxin M1 in milk and dairy products in different cities of Iran (Oveisi *et al*., 2007; Ghazani, 2009; Fallah *et al*., 2009), but there is no published data regarding aflatoxin M1 in raw milk produced in Ardebil City in north west of Iran and this study was carried out to evaluate the occurrence of aflatoxin M1 in raw milk produced in this region.

MATERIALS AND METHODS

Sample collection

A total of 122 samples of raw milk samples were randomly collected with in 6 months for the analysis of aflatoxin M1. The raw milk samples were obtained directly from cooling tanks of the milk collecting centers and dairy plants of Ardebil City before pasteurization. 30 mL milk was gathered and placed in an amber glass flask and kept refrigerated until analysis.

Sample examination and ELISA test procedure

The quantity of aflatoxin M1 was determined according to Enzyme-Linked Immuno Sorbent Assay (ELISA) method by using the Ridascreen® aflatoxin M1 (R-biopharm, Darmstadt, Germany) test kit which is a competitive enzyme immunoassay based on antigen-antibody reaction (Anonymous, 1999).

The milk samples were centrifuged for 10 minutes with 3500 rpm in 10°C. After centrifugation, the upper creamy layer was completely removed by aspirating through a Pasteur pipette. 100 µL of skimmed milk was used directly in each well for quantitative test. A sufficient number of micro titer wells were inserted into the micro well holder for all standards and prepared samples. Then 100 µL of the standard solutions and prepared samples were added in separate wells and they were incubated for 60 min at room temperature in the dark. The liquid was poured off the wells and the micro well holder was tapped upside down vigorously (three times) against absorbent paper to ensure complete removal of liquid from the wells. All the wells were filled with 250 µL of washing buffer and emptied as described earlier. The washing procedure was repeated twice. After that 100 µL of the enzyme conjugate was added and incubated for 60 min at room temperature in the dark.

The washing sequence was repeated three times. 50 µL of the substrate solution and 50 µL of chromogen solution were added to each well and mixed thoroughly. Then they were incubated for 30 min at room temperature in the dark. After this time, 100 µL of the stop reagent was added to each well and mixed completely. Finally, the measurement of AFM1 was done photometrically at the wavelength of 450 nm against the air blank in ELISA reader. Absorbance percentages were taken to the calibration curve performed with standards at different concentrations.

Statistical analysis

All statistical analyses were performed using the software SPSS, version 11.5. Analysis of variance ANOVA was employed after logarithmic conversion when necessary to detect significant differences among means. A probability level of $p<0.05$ was considered statistically significant.

RESULTS

In this study, a total of 122 raw milk samples were analyzed with the competitive ELISA technique. The occurrence and levels of aflatoxin M1 in raw milk samples produced in Ardebil City during six months are shown in Tables 1 and 2. Aflatoxin M1 contamination was detected in all of the examined raw milk samples. The aflatoxin M1 contamination levels were between 4 -112.4 ng/L with the mean of 40.01ng/L. There was a high incidence rate of aflatoxin M1 with 122 (100 %) milk samples being contaminated.

The mean of aflatoxin M1 contamination levels in October, November, December, January, February and March were 29.74, 40.69, 45.78, 46.13, 43.35 and 34.38ng/L, respectively. Also, the range of contamination levels varied among six months and they were ranged from 4 - 96 ng/L, 4.2 - 96 ng/L, 8.4 - 112.4 ng/L, 8 - 110 ng/L, 5.6 - 106.5 ng/L and 4.5 - 102.2 ng/L in October, November, December, January, February and March, respectively (Table 1). The lowest contamination was observed in October and the highest contamination was observed in January. The frequency of aflatoxin M1 contamination levels above 50 ng/L in October, November, December, January, February and March were 0.81, 1.64, 4.10, 4.10, 2.46 and 1.64 %, respectively (Table 2). In general, 18 out of 122 raw milk samples (14.75%) were beyond the

Table 1: Occurrence of aflatoxin M1 concentration (ng/L) in raw milk samples produced in Ardebil City during different sampling months

Months	N	Mean± [1]SE	[2]SD	Median	Range
October	27	29.74± 4.01	20.81	29.6	4 - 96
November	15	40.69 ±6.16	23.85	42	4.2 -96
December	15	45.78± 6.88	26.66	42	8.4 -112.4
January	20	46.13± 7.09	31.72	41	8 -110
February	19	43.35± 7.02	30.60	43	5.6 -106.5
March	26	34.38± 5.14	26.20	32	4.5 -102.2
Total	122	40.01± 6.05	26.64	38.27	4- 112.4

Table 2: Frequency distribution of samples and aflatoxin M1 levels (ng/L) in raw milk samples produced in Ardebil City during different sampling months

	Ranges of aflatoxin M_1			
Months	<10 ng/L *N (%)	11-25 ng/L *N (%)	26-50 ng/L *N (%)	50 ng/L < *N (%)
October	8 (6.56)	5 (4.1)	13(10.65)	1 (0.81)
November	2 (1.64)	3 (2.46)	8 (6.56)	2 (1.64)
December	1 (0.81)	3 (2.46)	6 (4.91)	5 (4.10)
January	3 (2.46)	5 (4.10)	7 (5.74)	5 (4.10)
February	4 (3.28)	2 (1.64)	10 (8.20)	3 (2.46)
March	9 (7.38)	4 (3.27)	11(9.02)	2 (1.64)
Total	27(22.13)	22 (18.03)	55 (45.08)	18 (14.75)

limit of ISIRI, Codex Alimentarius and European Community regulations. Also, results showed that in 104 samples (85.25%) the aflatoxin M1 concentrations were less than 50 ng/L and in 27 samples (22.13%) aflatoxin M1 was found less than 10ng/l. In 77 samples (67.11%), the concentrations were above 10 ng/L and less than 50 ng/L.

DISCUSSION

According to results obtained in this study, aflatoxin M1 was found in 100% of raw milk samples with 14.75% of the samples were higher than the permissible level of 50 ng/L as accepted by Iranian food standards (ISIRI, 2002; ISIRI, 2005) and European Community. With a view of the fact that milk is used by all the age groups including infants and children in Ardebil City, even the low amount of aflatoxin M1 in milk can be a serious public health problem. Since the commission of the European communities stated that even if aflatoxin M1 is regarded a less dangerous genotoxic carcinogenic substance than Aflatoxin B1, it is necessary to prevent the presence in milk and consequently in milk products, intended for human consumption and for young children in particular (Prandini *et al*., 2009).

Other researches have previously worked on the prevalence of aflatoxin M1 in milk samples (Table 3). The rate of aflatoxin M1 contamination levels obtained from our study is higher than the aflatoxin M1 levels in examined raw milk samples from Urmia (Tajik *et al*., 2007) and it is lower than aflatoxin M1 levels in reported raw milk samples from Sarab (Kamkar, 2005) and Tehran (Karim, 1998; Oveisi *et al*., 2007). It has been indicated that in many countries of Europe showed relatively low levels of aflatoxin M1 have been found in milk and milk products (Trucksess, 1999). The occurrence of aflatoxin M1 at such low levels in European countries may be the result of stringent regulations of aflatoxin B1 in complementary feedstuffs for dairy cattle. In many Asian countries and in Iran no severe control method for aflatoxin M1 in milk and milk products has been applied. In an Indian study of 87 liquid milk samples, incidence of contamination of aflatoxin M1 was of the magnitude of 87.3%. Almost 99% of the contaminated samples exceeded the European Communities and Codex Alimentarius recommended limits (EC, 1992; Shipra *et al*., 2004). Also, some studies have been done about aflatoxin M1 contamination in milk in other Asian countries like Kuwait, Pakistan and Syria. In a study, Dashti *et al*. (2009) reported that all fresh milk samples except one from Kuwaiti markets were contaminated with aflatoxin M1 ranging from 4.9 to 68.7 ng/L and eight samples exceeded the EC regulatory limit. In another research, Hussain *et al*. (2008) reported that all of the examined raw milk samples from fourteen districts of the Punjab province, Pakistan, were contaminated with aflatoxin M1 and 99.4% samples exceeded the European Union limit (0.05 μg/L). Also, Ghanem and Orfi, 2009 showed that 80% of tested raw milk samples collected from the Syrian market were contaminated with various levels of aflatoxin M1 ranging from 20 - 765 ng/L and the percentage of aflatoxin M1-

Table 3: The occurrence of aflatoxin M1 in raw milk samples in other studies

Location		Number of milk samples	Percent of contaminated milk samples above 50ng/L	References
Argentina		56	0.0	Lopez *et a*l., 2003
Greece		81	0.0	Markaki and Melissari, 1997
Greece		166	1.8	Roussi *et al.*, 2002
Indonesia		342	21	Henry *et al*., 2001
Italy		296	1.7	Decastelh *et al*., 2006
Philippiens		134	37.0	Henry *et al*., 2001
Thailand		310	84	Henry *et al*., 2001
UAE		59	56	Henry *et al*., 2001
Babol		40	56.7	Sefidgar *et al*., 2008
Iran	Tehran	73	82.2	Karim, 1998
	Tehran	100	78	Oveisi *et al*., 2007
	Sarab	111	40.0	Kamkar, 2005
	Urmia	72	12.5	Tajik *et al*., 2007
	Ardebil	122	14.75	Present study

contaminated samples exceeding the European tolerance limits was 52%. In other study, Bakirci (2001) found that 87.77% of the examined raw milk samples from the dairy plant in Turkey were contaminated with aflatoxin M1 and 44.30% of the positive samples were higher than the maximum tolerance limit (0.05 ppb) accepted by Turkey and European Union. The percentage of contaminated raw milk in present study is less than above mentioned studies in other countries.

Bilandzic *et al*. (2010) reported that the maximum mean concentrations of aflatoxin M1 recorded in winter-spring season were in the range of 35.8–58.6 ng/L and in summer–autumn season in the range of 11.6–14.9 ng/L and examined milk samples in winter–spring season have high levels of aflatoxin M1.

The aflatoxin M1 levels of our study in all months of October, November, December, January, February and March were higher than Iranian and European tolerance limits(50 ng/L), but many contaminated samples with high levels of aflatoxin M1 were found in months of December and January. This variation can be related to dairy feed quality. Because the quality of fodder used for feeding the cattle due to aflatoxin M1 in milk is important and the ingestion of food contaminated with aflatoxin B1 by dairy cattle is the way of exposure to aflatoxin M1 in milk (Lopez *et al*., 2003).

Many factors may affect the formation of aflatoxins in animal feeds. Geographic and climate changes can affect the farm management practices and feed quality. These effects can lead to the wide variations in AFM1 levels in milk (Ghazani, 2009). The preserved fodder such as silage and hay might have been contaminated by aflatoxin producing fungi and the improper storage led to aflatoxin production. The level of aflatoxin M1 in feed in rainy seasons is more than in dry seasons. It can be also probable to use higher amounts of contaminated concentrates in the cold months. Tajkarimi *et al* (2008) reported that the high levels of aflatoxin M1 contamination can occur in the cold months. The higher levels of aflatoxin M1 in this study obtained from cold months (e.g. December, January and February), which was reported by previous studies (Tajkarimi *et al*., 2007; Tajkarimi *et al*., 2008). Dairy cattle feed stuffs should be kept away from aflatoxin contamination as much as possible. It is important to maintain control and to apply an ideal recommended limit to minimize the health hazard from aflatoxin M1 contamination in milk which it can be used by infants and children. About this, governments have responsibility for making regulations to protect consumers against harm arising from chemical in milk. Government and producer must apply some methods and plans for prevention and control of aflatoxin M1 in milk and dairy products. About this, application of the Good Agricultural Practices (GAP) and Good Veterinary Practices (GVP) by agriculture and also the Hazard Analysis and Critical Control Point (HACCP) system as a draft code of practice for preharvest and postharvest control of dairy cow's feed and in milk and dairy products processing is effective. Responsibility for aflatoxin M1 control in milk and dairy products lies with all participitants in the production process, from farmers through to consumers.

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