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Original Article

A survey of aflatoxin M1 in cow milk in Southern Iran

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ABSTRACT

The competitive enzyme-linked immunosorbent assay technique was used to evaluate aflatoxin M1 (AFM1) levels in 168 samples of raw milk (135 samples and 33 samples from bulk tanks of farms and milk collection centers, respectively) and 12 samples of pasteurized milk in Fars province, Southern Iran. AFM1 was found in 55.56% of the samples with a mean concentration of 21.31 ng/L. The concentration of AFM1 in raw milk samples from farms was significantly ($p < 0.05$) lower than that in samples from collection centers and pasteurized milk. The concentration of AFM1 was not influenced by season, location, or type of farm. The concentrations of AFM1 in all samples were lower than the Iranian national standard limit (100 ng/L), but in 30% of raw cow milk samples they were higher than the maximum tolerance limit accepted by the European Union (50 ng/L); therefore, more effort is needed to control AFM1 levels in milk produced in Southern Iran.

1. Introduction

The presence of aflatoxins in food and feed is of great concern worldwide because of the health issues they can cause [1]. Aflatoxins are produced mainly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, and rarely by *A. nominus*, *A. tamarii*, or *A. pseudotamarii* strains when temperatures are between 24°C and 35°C and moisture content exceeds 7% [2–4]. Among the aflatoxins (B1, B2, G1, and G2), aflatoxin B1 (AFB1) is the most prevalent and potent natural carcinogen [5]. The presence of AFB1 in feeds and the subsequent access of lactating animals to it lead these animals to metabolize it to 4-hydroxylated form in their liver and excrete it as aflatoxin M1 (AFM1) in milk, urine, and feces [6,7]. About 0.3–6.2% of AFB1 in animal feeds is converted to AFM1, and it can be found in milk 12 hours after first ingestion and decreases to an undetectable level 72 hours after last ingestion of AFB1 [8,9].

Although previously AFM1 was assigned to group 2B (agents that are possibly human carcinogens) by the International Agency for Research on Cancer [10], it was thereafter reassigned to group 1 (class of agents that are certainly human carcinogens) for demonstrated toxic and carcinogenic effects [11]. A review of the literature shows that aflatoxins are most commonly known for causing acute or chronic liver disease depending on the doses used, but they are also considered immunosuppressive, hepatotoxic, mutagenic, teratogenic, and carcinogenic [2,12].

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Milk is one of the main foodstuffs in human diet especially for infants and children. Most studies indicate that processes such as pasteurization, sterilization, evaporation, concentration, or drying do not cause an appreciable change in the concentration of AFM1 in the product [7]. The AFM1 level in milk may vary according to geographic location, development level of the country, and climatic conditions; thereupon, it is important to determine its levels in produced milk in different locations to protect consumers from its harmful effects [13]. The maximum limits for AFM1 in raw milk vary in different countries depending on risk assessment and economic considerations. In the European Union (EU), the maximum level of AFM1 in liquid milk has been prescribed as 50 ng/L, whereas for United States and most of Asian countries' regulations it is 500 ng/L, which is higher than the maximum permissible level of 100 ng/L set by the Institute of Standards and Industrial Research of Iran [14–17].

Enzyme-linked immunosorbent assay (ELISA) is the quickest and simplest method for monitoring AFM1 in milk with good sensitivity, high precision, and optimal recovery [18].

The presence of AFM1 in milk has been shown in several surveys conducted in different regions of Iran using thin layer chromatography [19,20], high-performance liquid chromatography [21–23], or ELISA [24–34], and also in different countries worldwide: Brazil [13], Portugal [35], Spain [36], Lebanon [37], Syria [38], Turkey [39–42], Pakistan [43–45], South Korea [46], Sudan [47], Egypt [48], Morocco [49,50], Thailand [51], Indonesia [52], India [53], China [54], Serbia [1,55], and Croatia [56,57]. However, no published research is available on AFM1 levels in produced raw milk in Fars province. Annually, 497,000,000 L of milk is produced in Fars province, which ranks fifth in the country and first in the southern provinces of Iran [58]. The objective of this study was to determine the level of AFM1 in produced raw milk and to investigate its geographical and seasonal difference in Fars province (south of Iran).

2. Methods

2.1. Study area

A total of 192 milk samples were collected from three different areas in Fars province and labeled Sh, M, and S for Shiraz, Marvdasht, and Sepidan districts, respectively. Raw milk of cows from smallholder farms has was collected by milk collection centers, whereas it was transported to dairy factories directly by industrial dairy farms in Fars province. In each of these areas, raw milk was sampled from the bulk tank of three industrial dairy farms, three milk collection centers, and nine smallholder dairy farms (3 smallholder dairy farms that sold their milk to selected milk collection centers) seasonally. In each season, three pasteurized milk samples produced by dairy factories in Fars province were taken.

2.2. Milk sample preparation

Fresh milk samples (500 mL) were taken directly from storage tanks of farms or milk collection centers and pasteurized milk samples were bought from supermarkets. These samples were transported to the laboratory in ice boxes and stored in the dark at −18°C until the time of analysis. Milk samples were chilled at 10°C, of which 2 mL was centrifuged for analysis at 3500 rpm for 10 minutes at 4°C. As aflatoxins are water-soluble compounds [59], the upper creamy layers were completely discarded, and the lower phases were used for the quantitative test.

2.3. AFM1 measurement

The quantitative analysis of AFM1 was performed by competitive ELISA using an AFM1 kit (RIDASCREEN; R-Biopharm AG, Darmstadt, Germany). It had the following characteristics: detection limit, 5 ng/L; recovery rate, 95%; cross-reactivity, AFM1 100% and AFM2 30%; standard solutions, 0, 5, 10, 20, 40, 80 ng/L. The basis of the test was the antigen–antibody reaction. The wells in the microtiter strips were coated specific to AFM1 and filled with 100 μL of prepared samples or standard solutions. Antibodies were proportionally bound by shaking the plate gently and incubating at room temperature for 30 minutes in the dark. The wells were filled with 250 μL washing buffer after the complete removal of liquids. Then washing buffer was poured out, and this washing step was repeated twice. In the next step, 100 μL peroxidase conjugated AFM1 was added to the wells. Free antibodies were bound by conjugated AFM1 and any unbound enzyme conjugated AFM1 was removed by a washing step. Then, 100 μL of substrate and chromogen was added to wells and mixed gently by shaking the plate manually and incubated at room temperature for 15 minutes in the dark. Colorless chromogen was converted to blue by bound enzyme conjugate. Finally, 100 μL of 1N H_2SO_4 was added to wells, which led to a color change (from blue to yellow) [37]. The absorbance was measured at 450 nm in an ELISA plate reader (BioTek, Winooski, VT, USA). The absorption intensity was inversely proportional to the AFM1 concentration in the sample. A special software (RIDA SOFT Win; R-Biopharm AG) was used to draw standard curve and evaluate assays. The considered limit for positive samples was 5 ng/L AFM1.

2.4. Statistical analysis

All statistical analyses were carried out in SPSS for Windows 16.0.0 (SPSS Inc., 2007, Chicago, USA). Data were analyzed descriptively in the first step. Univariate analysis of variance was applied with AFM1 values as dependent variable and season, city, and herd type as independent variables. The means of AFM1 values was compared by using Duncan test. The relationship between contamination percentage and season or location in each type of farms was investigated using the chi-square test.

3. Results

Twelve raw milk samples were missed, and only 168 samples of raw milk (135 and 33 from bulk tank of farms and milk collection centers, respectively) and 12 samples of pasteurized milk were analyzed for AFM1. An exponential correlation was obtained by plotting the percentage of absorbance (y) and concentration (x) of AFM1 ($y = 96.72 - 10.2x$, with $R^2 = 0.991$) on

Table 1 – Mean ± standard error (SE), minimum (Min), and maximum (Max) aflatoxin M1 levels (ng/L) in raw and pasteurized milk samples.

Type of milk sample	No. of samples	Mean ± SE	Contaminated samples, *n* (%)	Min	Max	Exceeding limit,[a] *n* (%)
Raw milk						
Farm	135	18.26 ± 2.29[b]	64 (47.41)	0.00	99.92	20 (31.25)
Collection centers	33	29.82 ± 5.04[a]	25 (75.26)	0.00	97.68	8 (32.00)
Pasteurized milk	12	32.23 ± 6.76[a]	11 (91.67)	2.04	90.01	2 (18.18)
Total	180	21.31 ± 2.03	100 (55.56)	0.00	99.92	30 (30.00)

Means followed by different letters (a, b) are significantly different ($p < 0.05$).
[a] European Union limit (50 ng/L).

Table 2 – Mean ± standard error (SE), minimum (Min), and maximum (Max) aflatoxin M1 levels (ng/L) in raw milk of farms in different seasons, cities, and farms.

	No. of samples	Mean ± SE	No of contaminated samples, *n* (%)	Min	Max	No of samples above limit,[a] *n* (%)
Season						
Spring	34	15.55 ± 4.10	17 (50.00)	0.00	95.04	5 (29.41)
Summer	32	21.45 ± 5.68	11 (34.38)	0.00	92.48	7 (63.64)
Fall	34	21.16 ± 99.92	17 (50.00)	0.00	99.92	7 (41.18)
Winter	35	15.16 ± 78.66	19 (54.29)	0.00	78.99	1 (5.26)
City						
Shiraz	47	19.01 ± 4.01	21 (44.68)	0.00	92.48	8 (38.10)
Marvdasht	47	15.37 ± 3.79	19 (40.43)	0.00	99.92	4 (21.05)
Sepidan	41	20.73 ± 4.13	24 (58.54)	0.00	86.45	8 (33.33)
Type of farm						
Smallholder	99	18.42 ± 2.66	47 (47.47)	0.00	99.92	15 (31.91)
Industrial	36	17.82 ± 4.52	17 (47.22)	0.00	86.45	5 (29.41)

[a] European Union limit (50 ng/L).

the standard curve. The detection limit was found to be 5 ng/L. The results of analysis of milk samples for AFM1 are shown in Table 1. Although 55.56% of samples were contaminated with AFM1, the concentrations were not higher than the maximum tolerance level of AFM1 in liquid milk based on the Iranian national standard (100 ng/L) and Food and Drug Administration standard (500 ng/L) [14,16]. The overall mean level of AFM1 in the samples was 21.31 ± 2.03 ng/L, and 30 (30%) of the contaminated samples had AFM1 levels higher than the maximum tolerance limit accepted by the European Union [15]. Pasteurized cow milk showed a high rate (91.67%) of contaminated samples, with a mean AFM1 level of 32.23 ng/L, but only 18.8% of the samples were higher than the permissible level of 50 ng/L as accepted by the EU. The concentration of AFM1 was not influenced by season, location, or type of farm (Table 2). The distribution of contaminated samples in different seasons or cities in smallholder and industrial farms are shown in Figures 1 and 2. The chi-square results showed no relationship between contamination percentage and season or location in smallholder farms. Unlike location, season had a significant ($p < 0.05$) effect on the distribution of contaminated samples, and no sample showed contamination above 50 ng/L in spring and winter in industrial farms.

4. Discussion

As AFM1 is a global problem, many studies have been conducted for determining the occurrence and levels of AFM1 in milk using different techniques worldwide. The results of some of the studies that used ELISA to measure AFM1 are summarized in Table 3. The mean levels of raw

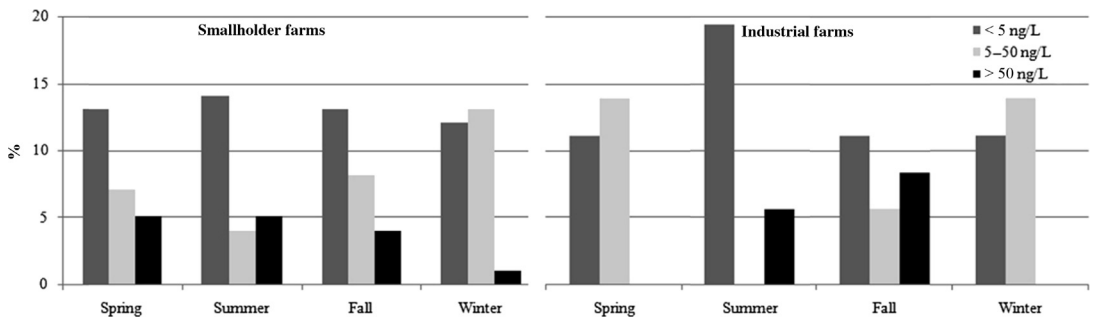

Figure 1 – Distribution of contaminated samples in different seasons.

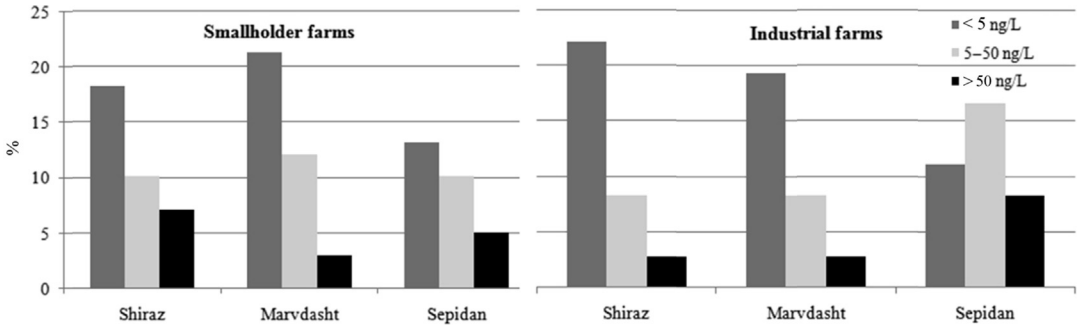

Figure 2 – Distribution of contaminated samples in different regions.

milk AFM1 in the present study was near to that obtained in Brazil [13], eastern part of Croatia [56], and Spain [36], but lower than that reported in other parts of Iran [25,26,28,32,33], India [53], Serbia [1], Syria [38], Turkey [42], and Lebanon [37]. These differences are probably attributable to variations in the amount of AFB1 in feedstuffs that dairy cows consume. Local weather conditions during preharvest and harvest stages as well as inadequate storage conditions can influence the quality of feed. AFB1 is produced by some molds that can easily grow in feeds having a moisture content between 13% and 18%, and environmental moisture between 50% and 60% [60].

Table 3 – Summarized results of studies on AFM1 contamination in cow milk by ELISA in different countries.

Country	Type of milk	No. of samples	No. of samples positive (%)	Mean (ng/L)	Range (ng/L)	No. of samples above limit (%)[a]	Reference
Brazil							
MG	Raw	129	129 (100.00)	19.50	0.2–106	18 (13.95)	[13]
Croatia							
Eastern part	Raw	194	47 (24.23)	20.60	3.7–162.3	13 (27.66)	[56]
Other parts		143	12 (8.39)	12.10	2.7–44.9	0 (0.00)	
India	Liquid	12	4 (33.33)	86.00	28–164	3 (75.00)	[53]
Indonesia							
Yogyakarta	Raw	113	65 (57.52)	8.53	No report	0 (0.00)	[52]
Iran							
Ahvaz	Raw	75	59 (78.67)	60.10	No report	27 (45.76)	[25]
Ardabil	Mix[b]	90	90 (100.00)	37.23	2.9–85	30 (33.33)	[26]
Hamedan	Raw	186	119 (63.98)	43.40	10–410	14 (11.76)	[28]
Gilan	Raw	90	56 (62.22)	No report	2.1–131	28 (50.00)	[29]
Ilam	Raw	54	34 (62.98)	43.98	10.03–85.24	31 (57.40)	[33]
	Traditional	48	19 (39.60)	34.21		8 (16.50)	
	Industrial Pasteurized	52	10 (23.80)	36.06		6 (14.28)	
Mashhad	Pasteurized	42	41 (97.62)	23.00	6.4–71.4	3 (7.32)	[30]
Qazvin	Raw	288	163 (56.60)	90.00	10–250	113 (69.33)	
Sanandaj	Raw	240	226 (94.17)	12.65	0.01–115.9	10 (4.42)	
	Pasteurized	32	31 (96.88)	12.43		2 (6.45)	
Shiraz	Pasteurized	624	624 (100.00)	No report	No report	101 (16.19)	[24]
Tabriz	Pasteurized	50	50 (100.00)	50.55	0–259	22 (44.00)	[34]
Tehran	pasteurized	128	128 (100.00)	72.20	31–113	100 (78.00)	[31]
Lebanon	Raw	38	28 (73.68)	60.40	2.63–126	17 (60.71)	[37]
	Pasteurized	25	17 (68.00)	30.60	3.27–84.4	4 (23.53)	
Spain							
Leon	Raw	92	5 (5.43)	20. 50	14–24.9	0 (0.00)	[36]
Serbia	Raw	678	540 (79.65)	282.00	No report	382 (70.74)	[1]
Syria	Raw	74	70 (94.59)	143.00	20–690	41 (58.57)	[38]
	Pasteurized	10	10 (100)	492.00	8–765	8 (80.00)	
Turkey							
Ankara	Pasteurized	85	75 (88.23)	No report	5.2–127.6	48 (46.00)	[41]
Kayseri	Raw	50	43 (86.00)	8.73	1–30	0 (0.00)	[40]
Kayseri	Raw	90	90 (100.00)	59.9	5–80	63 (70.00)	[42]

ELISA = enzyme-linked immunosorbent assay.
[a] European Union limit (50 ng/L).
[b] Raw, pasteurized, and sterilized.

The contamination rate of AFM1 in pasteurized milk in different parts of Iran was reported to be very high [24,27,30,31,34]. In Iran, pasteurization plants usually receive milk either directly from industrial farms or indirectly via milk collection centers without testing of milk for contamination with AFM1. The maximum levels of AFM1 in all samples were lower than the Iranian national standard limit (100 ng/L), which could be attributed to the activities of the Iran Veterinary Organization on testing and monitoring of raw milk in different locations of the country.

The concentration of AFM1 in raw milk from farms was significantly ($p < 0.05$) lower than that in milk samples from collection centers and in pasteurized milk. The raw milk produced in smallholder farms is collected and pooled with other milk during cooling in milk collection centers and then transported to pasteurization plants in Iran. No testing for contamination with AFM1 is done prior to receiving raw milk in milk collection centers. This leads to mixing of raw milk with different AFM1 levels, and subsequently elevating the level of contamination in transported milk to pasteurization plants. Many authors in Iran [19,20,26,32,33], Croatia [57], Serbia [1], and Turkey [39] reported higher AFM1 levels during cold seasons as compared to hot seasons, because stored feeds with a higher probability of containing AFB1 (e.g., dry hay, corn, concentrates, and silages) are used in much greater amounts for cow feeding during cold season, and this results in increased AFM1 content in milk. These differences are presumably attributable to variation in feeding systems. Dairy cows have been fed indoors without a grazing period during the year in Fars province. In conclusion, the results indicate that milk produced in Fars province is safe for human consumption according to the defined maximum tolerance level of AFM1 issued by the Iranian national standard. AFM1 concentrations exceeded 50 ng/L (maximum tolerance level of AFM1 in the EU) in 30% of samples; therefore, a more sustained effort is needed to control AFM1 level in milk produced in Fars province. The most effective way of controlling AFM1 is to monitor feed for AFB1. AFB1 can be controlled in animal feedstuffs by improving the production practices and using appropriate storage conditions. Dairy companies and milk collection centers should be required by relevant government organizations to test received milk for AFM1. The potential health risks of AFM1 may be reduced by enhancing the awareness of farmers, dairy producers, and consumers regarding the toxicity potential of aflatoxins.

Conflicts of interest

The author declare that he has no conflicts of interest.

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