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A Study on Contamination of Raw Milk with Aflatoxin M_1 at the Hamedan Province, Iran

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Abstract: A survey on the occurrence of aflatoxin M_1 (AFM_1) was carried out in the summer and winter using raw milk samples collected from 89 dairy farms situated in 8 cities in Hamedan province. All samples were analyzed for the presence of AFM_1 by competitive ELISA technique. AFM_1 was found in 185 out of 534 (34.6%) samples. The levels of AFM_1 in 14 (2.6%) samples were higher than the maximum tolerance limit (50 ng/L) accepted by European Union. Moreover, the difference due to cities under study was significant when considering the seasons($p<0.05$).That is, the mean concentration of AFM_1 was observed to be high during the winter months as compared with summer months in all the cities under study. The contamination ratio of milk in summer and winter months was 56.5 and 71.7%, respectively ($P < 0.05$). AFM_1 concentration greater than 50 ng/L has not been identified in any of the samples obtained during the summer while 15/7% of the samples obtained during the winter were higher than the maximum tolerance limit. Extrapolation of aflatoxin B_1 (AFB_1) from AFM_1 levels of contamination in milk samples indicated that the contamination in 5 (1.9%) dairy cattle feeds was higher than European Community limit (5μg/ kg). It is concluded that AFM_1 and Aflatoxin in feed stuffs should be controlled in the future.

Key words: Aflatoxin M1 · Raw milk · ELISA

INTRODUCTION

Aflatoxins (AFs) are extremely toxic substances produced by certain species of Aspergillus, especially *Aspergillus flavus, A. parasiticus*, and *A. nomius* that contaminate plants and its products. *A. flavus* produces only B aflatoxins while others species produce both B and G aflatoxins [1 - 3]. Several researchers reported that there is a linear relationship between the amount of AFM_1 in milk and AFB_1 in the feed consumed by the animals [4]. After the AFB_1 has been ingested, it is bio-activated by cytochrome P-450 to a genotoxic epoxide, a DNA reactive metabolite that forms N7– guanine adducts and it is also able to bind with proteins [5]. Milk and milk products are the most potent sources of aflatoxin among foods [6].

AFM_1 can be detected in milk 12–24 h after the first ingestion of AFB_1 [7]. Although the toxicity of AFM_1 is less than that of its parent compound, AFM_1 is known to be hepatotoxic and carcinogenic [8]. Therefore its toxicity, initially categorized by WHO–International Agency for Research on Cancer [9] as a Group 2B human carcinogen [10], has now been classified as Group 1 carcinogen [11]. The maximum level of AFM_1 allowed in liquid milk and dried or processed milk products varies from country to another and depends on economic considerations [12].

Many countries have carried out studies about the incidence of AFM_1 in milk and dairy products and proposed maximum levels for this mycotoxin [13, 14]. The European Community (EC) and the Turkish Food Codex (TFC) legal limit for AFM_1 in milk and cheese are 50 and 250 ng/kg, respectively [15, 16].

Many countries have conducted inspection program and controlling on mycotoxin for several years to promote public health. Some studies have been conducted in some provinces in Iran. No research is observed to be done in the western provinces [13, 17].

The aim of this study was to investigate the natural occurrence of AFM_1 in non-pasteurized milk obtained during the summer and winter from industrial dairy farms in the Hamedan province, which is an area of high-density livestock production in western Iran.

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MATERIALS AND METHODS

Sampling: A total of 534 samples were collected form industrial farms of Hamedan in the winter (January to March, 2009) and summer (July to September, 2009). The collected samples were transported to the laboratory in refrigerated containers (+4°C) and stored at -20°C until they were analysed.

Sample Preparation: Aflatoxins are water soluble [18] so the milk samples were centrifuged at 3000g for 10min at 10°C. After centrifugation, upper cream layers were completely discarded and the lower phases were frozen for the quantitative test.

Analysis of AFM_1 in Samples by Competitive ELISA: The quantitative analysis of AFM_1 in samples was performed by Competitive ELISA using I`screen AFLAM$_1$ test kit (Tecna S.r.l.Italy). It was stored at 2-8°C. Before its use the kit was left for 2h at room temperature to bring it to room temperature. The KIT was used according to the manufacturer`s instruction as follows: First add one hundred microliters of the AFM_1 standard solutions and test samples in each wells of microliter plate precoated with antibodies for AFM_1 and incubated for 45min at room temperature (20-25°C). Then the liquid was poured out of the wells and the wells filled completely with 250μl washing buffer and poured out the liquid again. This washing step repeated four times. In the next stage 100μl of enzyme conjugate were added to occupy the remaining free binding sites and incubated 15min at room temperature. At the end of incubation, the washing step was repeated. Then 100 μl of developing solution were added to each well and incubated for 15 min at room temperature. Using a multichannel pipet, 50 μl of the stop solution were added to each well and the absorbance was measured at 450nm in ELISA reader (ELX_800, Bio_Tek Instruments, USA).

Calculation of Extrapolated Values of AFB1 Concentration in Feeds: The values of AFB_1 in cattle feeds were extrapolated from back calculation of the values of AFM_1 obtained from analysis of milk samples. The calculation was based on the assumption that only1.6% of ingested AFB_1 is converted to AFM_1 by dairy cattle. AFB_1 level found in foods is calculated by the following equation [19, 20].

$$AFBI\ (\mu g/kg^{-1}) = \frac{AFMI(ng\ /\ kg^{-1} \times 100}{1.6 \times 1000}$$

Statistical Analysis: The results were analyzed by one-way analysis of variance (ANOVA) and considered statistically difference at 95% confidence levels.

RESULTS AND DISCUSSION

The standard curve for AFM_1 detection by competitive ELISA is given in Fig. 1. As can be seen from the figure, the calibration curve was found virtually linear in the 5–250 ng/L range. The detection limit was found to be 5 ng/L. The occurrence of AFM_1 in raw milk samples is shown in Table 1.

In this study, 534 raw milk samples were collected from 89 industrial dairy farms situated in 8 cities in Hamedan province in the winter (January to March 2009) and in the summer (July to September 2009). During the winter 267 samples were collected and the other 267 samples were collected in the summer from the same farm. AFM_1 was found in 185 (34.6%) of 534 raw milk samples examined. The levels of AFM_1 in 14 (2.6%) samples were higher than the maximum tolerance limit (50 ng/L) accepted by European Union [21]. AFM_1 concentration of 51 to 100 ng/L have not been identified in any of the samples obtained during the summer while 15/7% of the samples obtained during the winter were identified higher than the maximum tolerance limit (50 ng/ L) accepted by European Union which seems to be significant (Table 1). In addition AFM_1 was also identified in the rest of concentrations obtained in different months and season. In all the samples obtained the highest samples belonged to ≤5 concentration locking at different months during the seasons which show a descending trend in higher concentration (Table 1).

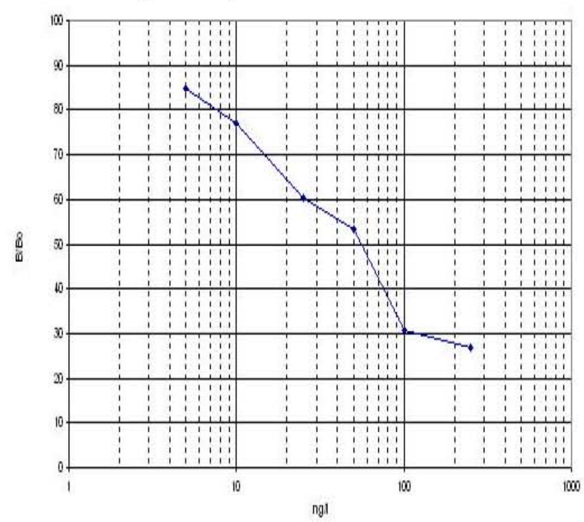

Fig. 1: Calibration curve of AFM$_1$

Table 1: Level of aflatoxin M1 in different month

Season	Month	Range of AFM_1 concentration (ng/L) ≤5	6-25	26-50	51-100	Positive samples**
Winter	July	*59(66.3)	27(30.3)	3(3.4)	0(0)	30 (16.2)
	August	68(76.4)	21(23.6)	0(0)	0(0)	21(11.4)
	September	64(72)	22(24.6)	3(3.4)	0(0)	25(13.4)
	Total	191(71.5)	70(26.2)	6(6.8)	0(0)	76(41)
Summer	January	56(63)	27(30.3)	2(2.3)	4(4.5)	33(17.8)
	Febrary	52(58.4)	28(31.5)	4(4.5)	5(5.6)	37(20)
	March	50(56.2)	21(23.6)	13(14.6)	5(5.6)	39(21)
	Total	158(59.2)	76(28.5)	19(7.1)	14(15.7)	109(58.9)
	Total sum	349(65.4)	146(27.3)	25(28.1)	14(2.6)	185(34.6)

Value in parenthesis indicate % of contaminated samples * **>5 ng/L afltoxin M_1

Similar results of AFM_1 contamination in milk-based products have also been reported in several European countries. In Italy, of 161 samples of dairy products, only 4 (2.5%) were contaminated at a level >50 ng/kg [13]. Roussi *et al.* [22] analysed 114 samples of raw and market milk in Greece and found 3 samples (2.6%) to be contaminated with AFM_1 > 50 ng/L. Grigoriadou, Eleftheriadou, Mouratidou, and Katikou [23] reported that in Thesaloniki Province levels of AFM_1 in milk were far below the tolerance level (highest value18.2 ng/L).

A recent survey on AFM_1 in pasteurized milk carried out in the south of Iran has shown that 17.8% of the samples had AFM_1 greater than 50 ng/L [24]. Recently published reports from Germany, Greece, Poland, Brazil, Italy and the U.K. almost all indicated that the levels ofAFM_1 were lower than 50 ng/L milk, probably as a consequence of stringent control of imported feeds in Europe [25]. Although some of the reports show only low or even no level greater than 50 ng/L [26 - 28], one exception was a study in India where 99% of contaminated samples had >50 ng/L [20]. Data on contamination of milk and milk products with AFM_1 have revealed that the highest levels were detected from Asian countries. Analytical results of milk samples taken from Indonesia and Thailand have shown that the highest AFM_1 levels were 23 and 6.6 μg/kg [29]. In Asia, high incidences and levels of AFM_1 at concentrations >0.05 μg/kg were found in the Philippines, Thailand, United Arab Emirates, Indonesia and Korea, i.e., 88, 84, 56, 37 and 37%, respectively [17, 29, 30, 31].

Irrespective of the method of analysis used, the main differences in the data from the aforementioned surveys may have been due to a large extent to differences in feed handling and storage practices. Countries that have legal limits and stringent regulations for AFM_1 have shown that the reduction of this toxin can be achieved by good manufacturing and storage practices of feed [32].

Seasonal variations of AFM_1 content of milk have been reported by some investigators [33, 34]; including one from Iran [14], but some reports such as a Grecian study [35] and a study in the U.K. [26] showed no seasonal influence on AFM_1 content of the milk samples. Several surveys in different countries, including Argentina, Brazil, Canada, Indonesia, Norway, the United Arab Emirates, the United State of America and several member states of the European Union, indicated a seasonal trend only in a few of them, with lower levels of AFM_1 in milk during summer months [29]. These seasonal variations, to some extent, may be due to feeding animals with fresh, less-contaminated feeds during summer. Storage conditions of feeds for winter, including temperature and humidity, as well as primary contamination of feeds with toxigenic fungi can be other possible explanations. It is important to emphasize that good agricultural handling and storage practices are required to minimize the risk of mold growth and mycotoxin contamination [36].

Mean of AFM_1 concentration in Hamedan cities considered together and separated during the winter and summer indicate a significant difference ($p<0.05$). As it can be seen, the highest AFM_1 concentration has been found in Razan and the lowest AFM_1 concentration has been observed in Hamedan. There figures range from a minimum of 5 to a maximum 16.7 in other cities (Table 2). Moreover, the difference in cities under study is significant ($p <0.05$) when considering the seasons. That is the AFM_1 concentration averages are observed more during the winter months as compared with summer months in all the cities under study (Table 2).

Table 2: Level of aflatoxin M1 in different month

	Maen ± 2 SD								
City	July	August	September	Total	January	February	March	Total	Total sum
Asad abad	12.6±6.1	8.5±5.5	11.5±6.6	13.8±8.2	20.3±8.6	25.1±13.1	16.9±5.7	19.1±7.05	16.4±7.6
Bahar	4.7±2.3	3.8±0.9	9.4±1.6	6.2±1.5	21.3±14.4	25.8±19.1	19.8±12.9	17.1±11.6	16.5±7.9
Hamedan	5.1±1.9	3.8±1.4	4.1±1.6	4.1±1.5	3.1±0.1	3.4±1.3	00±0.00	4.2±1.6	2.0±5.7
Kabodar Ahang	9.1±..6.0	6.9±4.9	7.4±5.4	7.3±5.1	10.5±7.8	14.9±12.4	7.8±8.9	4.2±2.2	3.6±1.3
Malayer	5.2±4.1	5.6±4.2	5.9±4.5	6.9±5.2	17.5±10.1	22.8±13.3	13.5±8.4	4.1±1.6	9.1±6.6
Nahavand	3.9±2.2	00±0.00	2.4±0.7	00±0.00	5.7±3.0	30.6±18.1	6.7±3.8	4.9±2.7	4.5±1.9
Razan	9.2±6.1	5.2±3.1	7.9±5.6	8.7±7.7	6/23±11.5	28.7±21.1	26.1±13.6	3.0±6.1	17.7±8.5
Toyserkan	2.1±0.71	00±0.00	1.8±0.5	2.4±0.8	15.1±13.8	12.7±13.4	10.9±15.1	6.9±9.7	12.6±7.2
Total	7.1±3.7	5.4±2.7	6.8±3.5	7.8±4.0	17.7±9.9	22.2±13.9	15.1±8.9	13.1±6.5	

* Maen ± 2SD of positive samples (containing >5 ng/L aflatoxin M1)

Table 3: Level of aflatoxin M1 in different month

	Range of AFB1concentration (μg/kg)		
Season	≤ 5	> 5	Total
Winter	262(98.1)*	5(1.9)	267(100)
Summer	267(100)	0(0)	267(100)
Total	529(99.1)	5(0.9)	534(100)

Value in parenthesis indicate % of contaminated samples*

Extrapolation of aflatoxin B_1 (AFB_1) from AFM_1 levels of contamination in milk samples indicated that contamination in dairy feeds was higher than 5μg/kg during the summer months while during the winter contamination in 5 (1.9%) dairy cattle feeds was higher than European Community limit (5μg/kg). That is, contamination in 0.9% of all the samples was higher than European Community limit (Table 3).

It seems that the kind of animal feeding and the harvesting time and temperature could be effective parameters in this regard. However, some studies indicated presumably as a result of toxin accumulation during storage under hot, humid conditions, often complicated by insect infestation [37]. Key elements are identified which can be used or modified to reduce mycotoxin formation in field and storage environments, such as limiting insect infestation and moisture levels in the commodities; removing immature and mold-infested kernels, grains or nuts; and physical and chemical inactivation and/or removal of the toxin [38].

One of the most influential factors on mold growth in animal feed is storage without adequate drying, which can lead to Aspergillus growth and aflatoxin production. Despite the present results, it is estimated that seasonal variation in some regions of Iran has an effective role on the fungus growth rate. Aflatoxin M_1 analysis and control must be taken seriously by the dairy industry. The AFM_1 level in milk is closely related to the mold contamination level in animal feed and other related factors.

To the best of our knowledge, this is the first report in AFM_1 contamination of Hamedan province milk products. The occurrence of AFM_1 in the present study was much higher than those reported from the Southwestern U.S.A. during 1995–2000 with 4.6% of samples with a concentration above 0.05 μg/kg, and 0.13% of samples with contamination levels exceeding 0.5 μg/kg [39]. However, contamination of 100% of milk samples has also been observed in some regions of Iran [24]. In conclusion, contamination of milk and its products with AFM_1 has the potential to be a public health problem in Iran, notably in Hamedan. Reducing the levels of AFB_1 in feed can be the first step in preventing the transfer of AFM_1 to humans and animals. In achieving this goal, it is necessary to set stringent regulations on AFB_1 in animal feed. The effectiveness of the animal feed regulations in keeping AFM_1 levels low in milk, by setting the levels of AFB_1 in cattle feed, has been demonstrated [32]. However, further studies on milk samples and other dairy products in other parts of the country, as well as over a longer period of time, are needed to estimate the representative intake of AFM_1 in Iran.

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