Aflatoxin M1 contamination of cow's raw milk in different seasons from Qazvin province, Iran

Peyman Ghajarbeygi[1], Marziyeh Palizban[1], Razzagh Mahmoudi*[1], Gholamreza Jahed Khaniki[2]

1- Department of Food Hygiene and Safety, School of Health, Qazvin University of Medical sciences, Qazvin, Iran

2- Environmental Health Engineering Department, School of Public Health, Tehran University of Medical sciences, Tehran, Iran

*Corresponding author email: r.mahmodi@yahoo.com

Abstract

Aflatoxins are extremely teratogenic, mutagenic, toxic, and carcinogenic compounds. In the present study, 60 cow's raw milk samples were collected from Qazvin province, Iran during Dec 2015 till July 2016.. Enzyme-linked immunoabsorbent assay (ELISA) was applied to determine Aflatoxin M1 (AFM1) in the milk samples. AFM1 was detected in 34 raw milk samples ranging from 6.25×10^{-3} to 127.87×10^{-3} (part per billion). AFM1 contents in all positive samples were far below the US legal limit (0.5 ppb), but AFM1 in30% of the raw milk samples exceeded the EU legal limit (0.05) and 5% of the samples exceeded the Iran legal limit (0.1 ppb). This study indicates a high occurrence of AFM1 in cow's raw milk especially in winter (40.71×10^{-3}ppb) but the level of contamination were not significantly different in different seasons ($P < 0.05$). Since contamination of milk with aflatoxin is a potential risk for human health, milk and milk products should be controlled periodically for aflatoxin contamination. The levels of AFM1 contamination of milk in the present study showed that the continuous examining the milk is necessary improving public health and reducing consumer exposure to aflatoxins. Reducing the levels of AFB1 in animal feedstuffs can be regarded as the initial step to control the transfer of AFM1 to the humans.

Keywords: Milk, Aflatoxin M1, ELISA.

1. Introduction

Mycotoxins are secondary metabolites produced by *filamentous fungi* (1,2). Almost 25% of food and food products are affected annually by mycotoxins (3). Among mycotoxins, aflatoxins are extremely toxic mycotoxins produced by three species of *Aspergillus (A. flavus, A. parasiticus, and rare A. nomius)* that contaminate plants and its products (4-7). *A. flavus* produces only aflatoxin B, while the others produce both B and G aflatoxins (6, 7). Under favorable conditions of temperature and humidity, aflatoxins can be produced during any stage of production including harvesting, storage, transport, and processing (5). When cows in their lactation period consume aflatoxin B1 contaminated feed, this toxin is metabolized to form the monohydroxy derivative, AFM1; which is appeared in the cow's milk (6, 8, 9). Previous studies have shown that approximately 0.3-6.2% of AFB1 ingested by livestock is metabolized into AFM1 and excreted in their milk however, it mainly depends on the genetics of animals, seasonal variation, milking process and the environmental conditions (2). The AFM1 is the main hydroxylated metabolites of AFB1 formed in liver by means of P450 cytochrome enzymes and may be found in milk products obtained from livestock that have ingested contaminated feed (6, 8, 9). The AFM1 derivative can be detected in milk within 12–24 hr after the first intake of AFB1, while its concentration decreases to an undetectable level 72 hr after the initial intake is stopped (9).

AFs are associated with the incidence of certain types of cancers which provokes a global concern over food safety (1). AFM1 carcinogenicity is approximately 2-10% that of AFB1 (4). The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has classified AFM1 as 2B group carcinogen (possibly carcinogenic to humans) (10) and this compound causes immunosuppressive, mutagenic, teratogenic and carcinogenic effects, which pose a health concern to humans (5, 11).

Mycotoxin detoxification processes of human food are still not efficient in terms of food safety, nutritional elements retention as well as cost (5). AFM1 is a very stable aflatoxin which neither storage (7, 11) nor thermal processing i.e. pasteurization, autoclaving, ultra-high temperature (UHT) or other methods used in the production of fluid milk, significantly affected its toxin(2, 11).

Since milk is an important part of human diet, especially for children; it appears that milk is one of the most important exposure factors to AFM1 (12). AFM1 is the only mycotoxin that has a legal limit in milk all over the world (13). The EU has implement the maximum AFM1 level in liquid milk and dried or processed milk products intended for adults i.e. 0.05 (ppb) and 0.025 (ppb) for milk intended for infants. However, the US Food and Drug Administration stated the maximum permissible level of 0.5 (ppb) in milk (2). Iran, have also conducted national

surveys to assess the AFM1 content in fluid milk in order to taking effective measures to ensure milk safety (table 1) (9).

The purpose of the present study is to evaluate the seasonal AFM1 contamination in raw milk distribution centers of Qazvin province for awareness AFM1 in milk (as the basis for the preparation of other dairy products); for provide health decisions at managerial level.

2. Materials and Methods

2.1. Sampling

This study was carried out from December 2015 to July 2016. A total of 60 cow's raw milk samples were collected in different seasons from raw milk distribution centers in Qazvin province (15 samples per season). Collected samples in Falcon tubes were transferred to the laboratory at 2-8 °C then frozen at -20 °C until examining for AFM1 contamination (14).

2.2. Examining milk samples for AFM1

An ELISA kit was used for the measurement of AFM1 in milk samples (5121AFMF [2]01.15, EuroProxima, kojast). Procedure was based on binding of free AFM1 in the samples and standard solutions to the anti-AFM1 antibodies during first incubation. For sample preparation, frozen samples were placed in a conventional refrigerator till samples were thawed and their temperature reached to less than 10 °C. At that time milk samples were centrifuged for 10 minutes at 3500g and the upper layer of the samples containing the fat were removed using Pasteur pipette. Then lower remaining liquid was used in ELISA test. An amount of 100 µl from each defatted milk samples were placed in separated 96 wells plate. 100 µl of the standards 1 to 6 were poured in micro wells. Gently rotating the plate, the plate was placed in a dark place for half an hour; at room temperature. Milk samples were discarded from the wells were and the wells were washed using washing buffer for three times. An amount of 100 µl of conjugated enzyme solution at 1 to 11 ratio, diluted in buffer No. 2; were added to each well and after gently mixing the plate was placed at a dark place at room temperature for 15 minutes. The content of the wells were discarded and the wells were washed three times using washing buffer. An amount of 100 µl of chromogenic substrate was added to each well and after mixing; the plate incubated for 15 minutes in a dark place at room temperature. To end the reaction, 100 µl stop solution was added to each well and for up to 15 minutes later, the absorption were read at 450 nm.

2.1. Statistical Analysis

The results of AFM1 concentration were statistically analyzed and the data were presented as mean and range. The significant difference ($p<0.05$) between provinces and lactation times (morning and evening) were determined by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc, IBM, NY, USA).

3. Results and Discussion

In this study a total of 60 milk samples were examined for detection of AFM1 contamination. Obtained results were summarized and shown in Table 2 (comparison based on ISIRI5925 Amendment No. 1).

The linearity of the standard calibration curve was gaged by calculating the coefficient of the regression curve (R^2), which stayed not under 0.999. 34 of samples from all seasons were contaminated with AFM1 in different levels. Analysis of 60 collected raw milk samples showed that the lowest percentage of samples contaminated with AFM1 belonged to spring (100% Corresponded to ISIRI5925 Amendment No. 1). The AFM1 maximum concentration (0.04071 ppb) observed in the winter. As moisture is one of the factors influencing fungal growth and aflatoxin production; the prevalence of aflatoxin in winter can be attributed to the high rainfall and high humidity in this season. Based on statistical analysis, aflatoxin levels of milk samples in four seasons were not significantly differed. Overall, three and 18 samples of whole samples were unsuitable for human consumption according to last Iranian Regulation and EU, respectively 117.30-127.87 ($\times 10^{-3}$ ppb) and 50.25-127.87 ($\times 10^{-3}$ ppb) (23,24).

According to the study of Jovana Kos in Serbia, AFM1 was detected in 98.7% of analyzed cow's milk samples with concentrations from 0.01 to 1.2 (ppb). In additional,129 (86.0%) cow's milk samples contained AFM1 in concentration greater than maximum residue levels (MRL) of 0.05 (ppb) defined by European Union (EU) Regulation(15).

In a Seasonal study of AFM1 contamination in milk by A. Fallah in Yazd, Iran; Levels of the toxin in 15.4% of cow milk, 11.5% of sheep milk, and 9.15% of goat milk samples surpassed the 0.05 (ppb) while none of the camel milk samples exceeded this limit (16).

Examining AFM1 in raw milk throughout four seasons in Croatia showed the mean AFM1 levels in the three regions over four seasons were in the ranges (ppb): eastern Croatia (7.25-26.6) $\times 10^{-3;}$ western Croatia (5.91-9.26) $\times 10^{-3}$; other regions of Croatia (7.17-13.6) $\times 10^{-3}$. The highest AFM1 levels were quantified in December (764.4×10^{-3} ppb) and January (383.3×10^{-3} ppb) (17).

In an assessment of AFM1 in milk consumed in Kosovo during 2009–2010, From 895 samples examined by competitive enzyme linked immune sorbent assay (ELISA) method, 25 (2.8%) samples were contaminated with AFM1, none of contaminated samples did not exceed the European Union regulation limits (0.05 ppb) (18).

In a study by Mahmoudi and Norian, 288 milk samples were randomly collected from individual farms in Qazvin province from March to February 2012, Iran. The mean AFM1 contamination levels in milk in summer and winter were 0.08 and 0.18 ppb, respectively. The AFB1 contamination level in winter feed (2.27 ± 1.76) was higher than from summer (0.83 ± 0.60) ($P < 0.05$) (19).

In a seasonal pattern study on AFM1 contamination in buffalo milk in the northwestern region of Iran, this mycotoxin was found in 54.4% of the samples by average concentration of 38.5±5.12 ($\times 10^{-3}$ppb). The concentration of AFM1 in all of the samples were lesser than Iranian national standard and FDA limit (0.5ppb), but in 16.3% of the milk samples the concentration of AFM was higher than maximum tolerance limit established by European Union/Codex Alimentarius Commission (0.05ppb)(20).

In a study examining a number of 144 milk samples (102 raw milk samples and 42 pasteurized milk samples), AFM1 was detected in 47.91% of the samples by average concentration of 39.45 ± 18.40 ($\times 10^{-3}$ppb). The highest mean concentration of AFM1 was recorded in traditional dairy farm samples [43.9 ± 9.5 ($\times 10^{-3}$ppb)] (21)(Table 3).

Traditional dairy farming is a common system in Iran, done mostly by farmers in a system of mixed farming, with animals in support of crop production. As a result, poor foodstuff quality might be related to the residual level of aflatoxin in the milk (22). However, the average AFM1 contamination of the milk samples in the present study was in agreement with the results of the previously mentioned studies, but there is no significant difference between samples from different seasons (Table 2).

4. Conclusion

The results of this study didn't revealed a relatively significant occurrence of AFM1 contamination in cow's raw milk. Contamination of milk samples with AFM1 could be regarded as a potential public health problem. Codex as an international organization is responsible for food-related regulations to facilitate traditional exchange and established AFM1 standard in milk about 0.05 (ppb)(2).

There is a seasonal trend of AFM1 contamination in the milk of cow, sheep, and goat, with higher occurrence and levels of the toxin during cold seasons (16). Appropriate measures for decontamination of animal feed in order to

preventing the production of fungal toxins in feedstuffs are essential. The government should have more supervision on traditional dairy farms; and these dairies should be gradually replaced by industrial ones.

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Table 1: Acceptable limit of AFM1 level in liquid milk (2.23, 24)

EU(ppb)	ISIRI(ppb)	FDA(ppb)
0.05	0.1	0.5

Table 2: Occurrence of AFM1 in cow's raw milk samples.

season	Date (month/year)	Sample category	No	Range of concentration (ppb) Min-Max	Mean[NS] (ppb)	n> ISIRI limit[a]
autumn	Dec/2015	Raw bulk milk	15	<0.00625-0.11929	0.03913	1(6.66%)
winter	Feb/2016	Raw bulk milk	15	<0.00625-0.11730	0.04071	1(6.66%)
spring	June/2016	Raw bulk milk	15	<0.00625-0.09381	0.03846	0
summer	July/2016	Raw bulk milk	15	<0.00625-0.12787	0.03417	1(6.66%)

[NS] Not Significant

Note: [a] Iran legal maximum level (0.1ppb).

Table 3: Comparative evaluation in term of AFM1 contamination between countries

Local of sampling	**year**	**Category of samples**	**Evidence of AFM1 (%)**	**Contamination upper than EU (%)**
Iran	2013	raw and pasteurized milk	47.91	0
Serbia	2014	raw cow milk	98.7	86
Iran	2014	raw buffalo milk	54.4	16.3
Kosovo	2016	raw cow milk	2.8	0
Iran	2016	raw cow milk	-	15.4

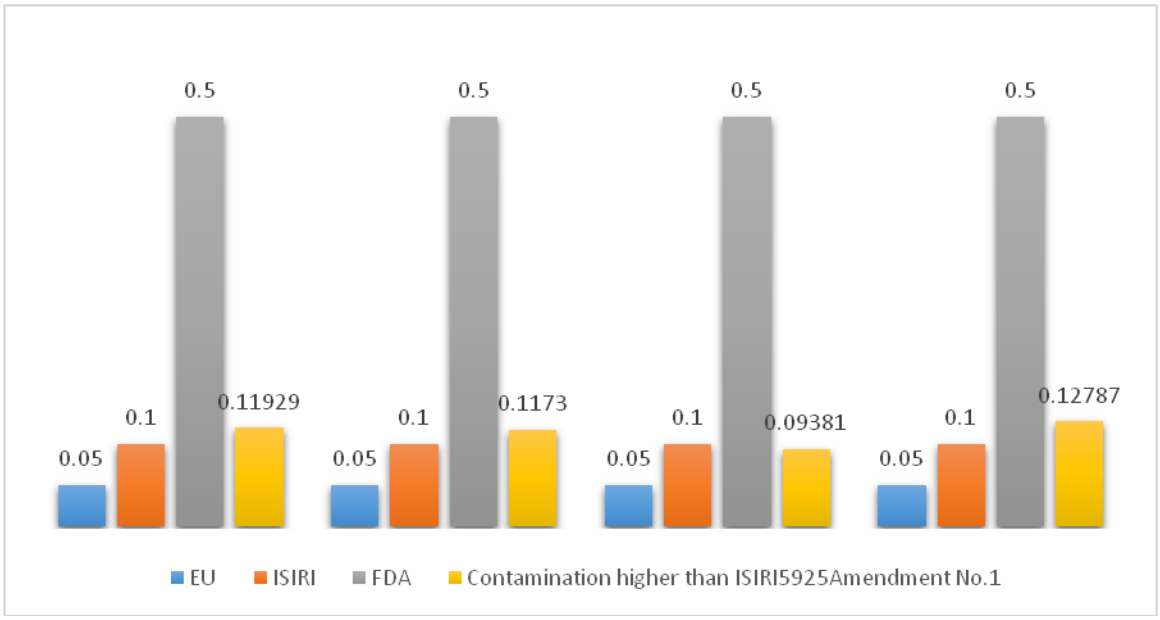

Figure 1: Comparison of the levels of AFM1 contamination by existing standards (ppb)

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