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Determination of aflatoxin M_1 levels in raw milk samples using ELISA and high-performance liquid chromatography in Qazvin, Iran

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Abstract:

Use of raw milk and dairy products containing aflatoxin M_1 (AFM_1) has led to concern in consumers. The present study determined the AFM_1 in raw cow's milk in Qazvin province. In this research, 170 raw cow's milk were collected from dairy farm, dairy factories, milk collection centers, and milk supply centers in Qazvin province during cold seasons in 2013, and all samples were examined for AFM_1. The samples were analyzed with a commercial competitive enzyme-linked immunosorbent assay (ELISA) kit and high-performance liquid chromatography (HPLC). AFM_1 contamination was observed in all milk samples. Fifty-seven milk samples (33.52%) had a contamination of AFM_1, higher than the threshold level of The Institute of Standards and Industrial Research of Iran (0.5 ng/ml), whereas in 113 milk samples (66.48%), concentration of AFM_1 was less than the limits permitted. The mean concentration of AFM_1 in dairy farm was 0.215 ng/ml, in dairy factories 0.268 ng/ml, in milk collection centers 0.734 ng/ml, and in milk supply centers 0.409 ng/ml. Because of high levels of contamination observed in samples, regular monitoring of contamination in milk samples and controlling most contaminating causes are necessary.

Keywords: aflatoxin M_1, ELISA, HPLC, milk.

Introduction

Mycotoxins are secondary metabolites of fungi that may be toxic, carcinogenic, mutagenic, and may cause malformation (Van Egmond, 1995). AFs are a group of highly toxic mycotoxins and are easily developed during growth and storage of food. Side effects of AFs usually appear in two forms: first, the instant effects because of poisoning and second the gradual effects that are related to carcinogenicity (Van Egmond, 1993). At least 17 types of aflatoxins are found in nature in which AF G1, G2, B1, and B2 are more important (Lopez *et al*., 2001). Shortly after the

discovery of AFs in food, authors suggested that AF residues may be found in milk and other products from animals that have been fed by food contaminated with AFs. So after removing the toxin from milk, it was named AFM_1 (Van Egmond, 1983). As shown in most researches, the conversion ratio of dietary AFB_1 to AFM_1 was reported to be 1-4% or 1-3% (Aycicek *et al*., 2005). However, conversion ratio up to 6% of the daily consumption of AFB_1 has been reported. Overall, it can be asserted that the most dangerous AFs is B1 that mainly converts to its 4-hydroxy derivative in liver microsomals with the interference of multifunctional oxidases in dairy cows, and eventually, AFM_1 is produced (D'Mello and Macdonald, 1997). Although no reliable method can guarantee the complete prevention of aflatoxin contamination of agricultural products, there are ways to detoxification, including decreasing the toxins, toxin structural damage, or inactivation of AFs (Samarajeewa *et al*., 1990).

Research results have shown that one of the ways to decrease the prevalence of aflatoxin-related disorders in milk, meat, and eggs is to reduce their amount with absorbent agents (Samarajeewa *et al*., 1990). Final evaluation of the permitted level of AFM_1 was determined by JECFA in 2001. Based on this assessment, the maximum possibility of cancer development and the highest per capita consumption of milk, according to the World Health Organization (WHO), and the amount of milk contamination are calculated to be 0.05-0.5 ng/ml. According to the FDA standards and the Codex Alimentarius standards, there are different methods for determination of aflatoxins, such as immunoassay and quantitative analysis methods. Immunoassay methods, for example, ELISA, are recommended for screening tests and monitoring the milk and its products in the factories. Sampling is very simple for the determination of AFs in milk, but the main problem is how to isolate this toxin from milk and its products (Bakirci, 2001; Ivastava *et al*., 2001; Martins *et al*., 2004). The important point in the use of Immunoassay method is to do the confirmation test using quantitative analysis methods like thin-layer chromatography (TLC) (Park, 2002; Rastogi *et al*., 2004) and high-performance liquid chromatography (HPLC) (Bakirci, 2001; Ivastava *et al*., 2001; Rodríguez Velasco *et al*., 2003). Because of the side effects and complications caused by AFM_1 residues in milk and dairy products, it is necessary to monitor raw milk periodically. The purpose of this study was to screen the amount of AFM_1 in raw milk produced in Qazvin province using ELISA and to confirm the results using HPLC in cases that were above the limit.

Materials and Methods

Sampling: In this study, 170 samples of raw cow's milk were collected from milk containers in dairy factories, industrial farms, milk collection centers, and other milk supplies in winter (January to February 2013). Each sample was calculated on the basis of a unit. Milk collection centers are generally cattle farms with less than 20 cows, and milk supply centers often include stores in which milk and other dairy products (such as yoghurt and ice cream) are sold on a daily basis. In the case of dairies and cowsheds, each factory or farm is considered a unit. One sample of (50mL) raw milk was taken from each unit and was eventually collected in a 50ml sterile container from SUPA Company along with dry ice and

within 6-8 hours (depending on the distance between the collection centers and the laboratory). The samples were transferred to the laboratory and were stored at −20°C until testing. Sampling was according to the National Standard Institute of Standards and Industrial Research of Iran's milk samples.

ELISA method: The quantitative analysis of AFM_1 in the milk samples was performed by competitive enzyme immunoassay using Euroclone Aflatoxin M_1 ELISA kit (Quantative Euro Clone Aflatoxin M_1, Cod. EEM005096. LOT. AM11110V).

Milk samples were prepared according to the manufacturer's instructions. Milk samples were kept at 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), 200µl was directly used per well.

ELISA was conducted according to the manufacturer's instructions. Two hundred microliters of standard solutions (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 (250µl) thrice. In the next stage, 200µl 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. Then, 200µl of substrate/chromogen was added, mixed gently, and incubated in the dark at room temperature for 15 min. Finally, 50µl 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.

HPLC method: Milk samples, which had the maximum residues limit (MRL) based on ELISA, were evaluated with the HPLC method for final approval. In this method, reverse-phase chromatography with fluorescence detector with excitation of 360 nm and an output of 2475 WATER at 440 nm was used. Required columns were ODS (Octadesyl Ceylon) with the dimensions of 6.4×250 mm and the protective column. The speed of mobile phase with 1525 WATER pump was 8 ml/min. Before performing the action, linearity of the calibration curve and the stability of chromatograph were checked. A constant concentration of AFs was injected to fix height and area under the curve, and it was obtained at a difference of ±5%. To draw the calibration curves, different and consecutive concentrations of 10, 7.5, 5, 2.5, 2, 1, 0.5, 0.2, 0.1 ng/ml of standard solution of AFM_1 (AF standards were purchased from Sigma Chemical Company, USA) were prepared and injected, and according to the following chart, calibration curves were drawn. Two hundred milliliters of the prepared solution was injected into the system. For data verification, a concentration of the calibration solution was administered after every 10 injections. The toxin concentration was measured as nanograms milliliter using the peak and the area under the calibration curve. The actual concentration of AFM_1 was determined in the positive samples (Mahmoudi *et al*., 2013).

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

Results

In this study, a total of 170 samples sourced from dairy factories, farms, milk collection centers, and centers of bulk milk supply were taken (12, 80, 23, and 55 samples, respectively), and to assess the presence of AFM_1, samples were first screened by ELISA. Average values of AFM_1 in farms, dairy factories, milk collection centers, and milk supply centers were 0.215 0.268, 0.734, and 0.409 ng/ml, respectively. The maximum contamination level of AFM_1 was found in the milk obtained from milk collection centers with the average of 0.734 ng/ml and the minimal contamination was found in the milk obtained from farms with an average of 0.215 ng/ml. The percentage of the highest contamination levels, which was higher than the permissible limit, was found in the milk samples obtained from bulk milk supply centers with 52.72% infection, and the minimal contamination was found in the milk obtained from dairy factories with 8.33% infection (Table 1).

Table 1. Number and frequency of AFM_1 in milk samples

Location of sampling	Samples size	Positive frequency	Positive percent	Average ± SD (ng/ml)	Lower limit (ng/ml)	Higher limit (ng/ml)	Count of higher limit (5 ng/ml)	Percent of higher limit (5ng/ml)
Dairy Factories	12	12	100	0.268±0.024	0.0281	0.486	1	8.33
Farms	80	80	100	0.215±0.053	0.0149	0.715	18	22.52
Milk collection centers	23	23	100	0.734±0.071	0.0347	1.118	9	39.13
Centers of bulk milk supply	55	55	100	0.409±0.067	0.0183	0.926	29	52.72
Total	170	170	100	0.4	0.023	0.811	57	33.52

The total amount of contaminated milk samples collected in winter (January) was 100%. In other words, varied amounts of AFs were detected in all the milk samples that were obtained from different sources. The amount of AFM_1 in all samples, which had the maximum residues limit (MRL) based on ELISA, was evaluated with the HPLC method for final approval. The results showed that 33.52% of the cases were higher and 66.48% of the cases were lower than the standard limits in Iran (0.5 ng/mL, Table 1).

According to the results, milk contamination with AFM_1 in some samples from milk collection centers was found to be more than 2.2 times the standard limit (0.5 ng/mL) (Fig. 1), and because the samples of milk collection centers are a good indicator to show the status of traditional farms, the amount of pollution is evidence to improper feed storage in rural communities in winter.

Discussion

Mycotoxins are biological compounds produced by molds that can affect food quality and manufacturing. Therefore, for the consumers' health, it is necessary to detect the presence and amount of mycotoxins in foods persistently and plan to minimize them in the food chain. Our results showed that 100% of the samples prepared were contaminated more or less by AFM_1. The

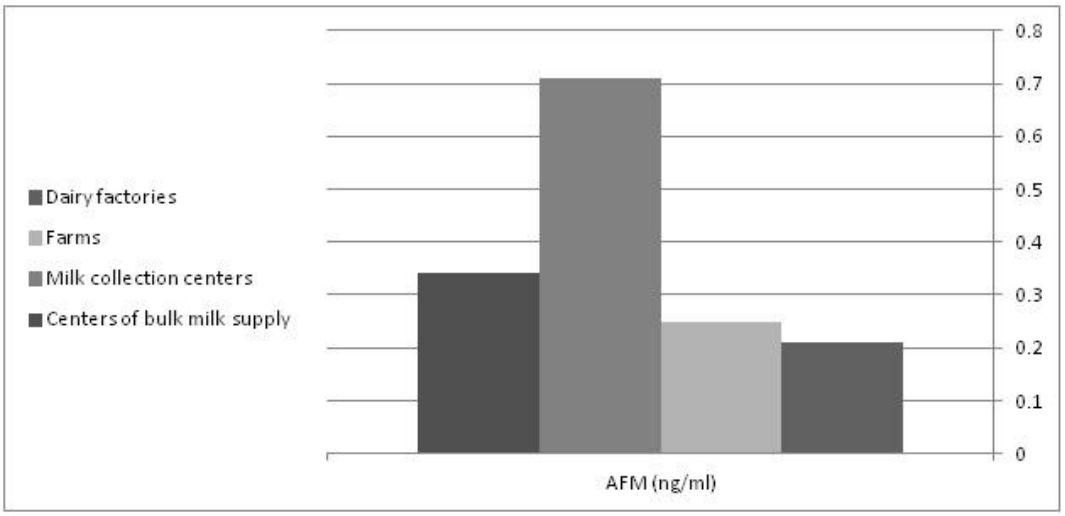

Fig. 1. Milk contamination with AFM_1 in different milk suppliers

highest contamination was found in the samples of milk collection centers with an average of 0.734 ng/ml, and the lowest contamination was found in the samples of farms with an average of 0.215 ng/ml. The percentage (52.72%) of the highest contamination levels, which was higher than the permissible limit, was found in the milk samples obtained from bulk milk supply centers, and the minimal contamination (8.33% infection) was found in the milk obtained from dairy factories. There are various reports about the prevalence of AFM_1 in milk in Iran and also in the present study. Previous studies in Iran in most cases have shown a high prevalence of infection. AFM_1 contamination was found in 82.2% of the milk samples in one study and 92.3% in another. The study by Tehran and Kamkar (2005) showed that 76% of milk samples that were tested were contaminated with mycotoxin.

Livestock feed contaminated by AFB_1 (precursor of AFM_1) is the major cause of AFM_1 contamination in milk, and the presence of AFM_1 in the forage indicates adequate conditions for mold growth and the production of mycotoxins. During winter, industrial and stored feed is usually used instead of fresh hay to feed dairy cows, which have higher possibility of fungal growth and AF formation, especially AFB_1 which can result in the presence of AFM_1 in milk. However, the potential risks of AFs for humans, particularly AFB_1 and M_1, in milk and agricultural products have been corroborated by several investigators (van Egmond, 1983). Risks to human health, particularly liver cancer, because of consumption of milk and dairy products have a great deal of importance. In Greece, Melissari and Markazi evaluated the amount of AFM_1 in the pasteurized milk of commercial shops using ELISA and HPLC. The amount of AF in 32 samples out of 81 samples was 2–2.5 ng/ml, 31 samples had 0.5-1 ng/ml, 9 samples had aflatoxin more than 5 ng/ml, and 9 samples had no AF (Markaki and Melissari, 1997).

In the study by Panariti, skimmed milk and semi-skimmed milk had less pollution than the whole milk. In the study by Gurbay, 59.3% of 27 milk samples tested by HPLC were contaminated from which only one sample exceeded the limit of Union Europe standard and the Codex Alimentarius

(Gurbay *et al*., 2006). In the study by Kamkar, 85 samples of raw milk out of 111 (76/6%) from Sarab, Iran, were contaminated with concentrations of 0.015–0.28 ng/ml and 40% of positive samples were above the limit of the Union Europe (0.05 ng/ml) (Kamkar, 2005). The results of a previous study in the western region of Iran showed that 59.72% and 36.11% of milk samples collected in winter and summer, respectively, were contaminated by AFM_1. The occurrence rates of AFM_1 in raw milk were 66.6% and 37.2% in winter and summer, respectively. Also, the occurrence rates for pasteurized milk samples were 42.8% and 33.3%. The mean concentration of AFM_1 in all milk samples in winter was significantly ($P < 0.05$) higher than that obtained in summer (Vagef and Mahmoudi, 2013).

Because of humidity and high temperature, it is difficult to prevent the AF formation in the diet before harvesting but proper storage of the feed can result in reasonable reduction of AF (Van Egmond, 1993).

Although the contamination rate of analyzed samples in this survey was higher than those reported in Iran and in the world, the percentage of samples above the legal limit was low (Beheshti and Asadi, 2014; Rahimi *et al*., 2008; Mahmoudi and Norian, 2015; Sassahara *et al*., 2005; Kangethe and Langa, 2009).

These differences can be explained by the diversity in analyzed feedstuffs, storage conditions, geographical areas, and climate conditions in different studies (Eskandari and Pakfetrat, 2014).

Variables such as sampling scheme, preparation of sample, and method of AFB1 detection should be considered when comparing the results (Sassahara *et al*., 2005; Rashid *et al*., 2012).

Milk and dairy products are one of the main sources of nutrition for human beings, especially children who are more sensitive to the effects of AFs, and their ability to change biological carcinogens is more slow than adults. These products may be contaminated and dangerous. Provisions are made regarding the reduction of mold contamination of animal feed. In this study, levels of aflatoxin in milk are high, and this is a serious public health problem. Milk and milk products must constantly be evaluated, at least twice a year for AFM_1 contamination. Low amount of AFB_1 is also important in milking animal feeds. To achieve this goal, it is necessary for the organizations associated with milk production, particularly veterinary organization, to take special measures and educate the producers to improve the quality and reduce the amount of mycotoxins and pesticides in raw milk. It is also necessary to examine the contamination of raw milk to AFM_1. Contaminated products should be eliminated after detection. Animal feeds should be checked regularly for AFB_1, and storage conditions of feeds must be taken under strict control.

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