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Detection of Aflatoxin M1 in Raw and Commercial Pasteurized Milk in Urmia, Iran

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Abstract: During the years 2005 and 2006, samples of raw and of pasteurized milk (72 samples each) were collected randomly from various parts of Urmia city in Iran for the detection of aflatoxin M1. Aflatoxin M1 levels were assessed by Enzyme Linked Immuno Sorbent Assay (ELISA). There was a high incidence of AFM1 (100%), in both raw and pasteurized milk samples. The AFM1 levels in 6.25% of samples were higher than the maximum tolerance limit accepted by European Union (50 ng L^{-1}), while the observed mean of AFM1 was lower than those proposed for European diets. Maximum level of AFM1 in raw and pasteurized samples were 91.8 and 28.5 ng L^{-1}, while minimum levels were 4.3 and 5.1 ng L^{-1}, respectively. The levels of AFM1 in total samples indicated that feeds for cows in this region were contaminated with AFB1 in such a level that appears to be a serious public health problem at the moment. Therefore, there is a need to limit exposure to aflatoxins by imposing regulatory limits.

Key words: Aflatoxin M1, milk, ELISA

INTRODUCTION

Aflatoxins are fungal metabolite that contaminates the food supply in certain areas of the world (Cathey *et al*., 1994; Smela *et al*., 2001). They are produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, which grow on improperly stored foods. The main sources of aflatoxins in feeds are peanut meal, maize and cottonseed meal (Singh *et al*., 1996). Aflatoxin M1 is a toxic metabolite of aflatoxin B1. It is formed by enzymatic hydroxylation at the 9α-position in the livers of animals and humans following the ingestion of aflatoxin B1-contaminated feed, primarily cereal grains. It is normally excreted in the urine and also in the milk of dairy cattle (Cathey *et al*., 1994; Gurbay *et al*., 2006; Oliveira and Ferraz, 2007). The metabolite present in the milk of nursing women who eat foods containing the toxin (Henry *et al*., 2001). The occurrence of aflatoxin M1 in milk is transitory in nature, usually reaching a peak within two days after the ingestion of the contaminated commodity they are disappearing within 4-5 days after the withdrawal of the contaminated source (Henry *et al*., 2001). Although AFM1 is less carcinogenic and mutagenic than AFB_1, it exhibits a high level of genotoxic activity and certainly represents a health risk because of its possible accumulation and linkage to DNA (Shundo and Sabino, 2006). Major concerns with aflatoxins are their potent carcinogenic, mutagenic and teratogenic effects in susceptible laboratory animals and their acute toxicological effects in humans (Battacone *et al*., 2003; Cathey *et al*., 1994).

According to the International Agency For Research on cancer (IARC), AFB_1 and AFM1 categorized as a class 1 and class 2B carcinogen, respectively (Kamkar, 2005; Rastagi *et al*., 2004). This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer (Eaton and Groopman, 1994). Many methods of analysis have become available for the determination of aflatoxin M1 in milk and milk products, both for screening and for quantitative estimates. Most were developed for the analysis of milk and milk products, with minor modifications (Henry *et al*., 2001). Although analytical methods might consist of different extraction, clean up and quantitation steps, the results of the analysis by such methods should be similar when the methods are applied properly. Radioimmunoassays have found little application as a screening test, whereas Enzyme linked immunosorbent assay (ELISA) are often used in routine investigation of aflatoxin M1 in milk (FAO/WHO, 2002).

Numerous studies have been conducted on the effects of processing on the concentration of aflatoxin M1 in milk, the results of which were variable. Most studies

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show that the concentration is not appreciably reduced by heat treatment or by processing yogurt, cheese, cream, milk powder and butter (FAO/WHO, 2002). Due to the high toxicity of aflatoxin M1, the European Community (EC) established the maximum level of 0.05 μg kg^{-1} for aflatoxin M1 in liquid milk. This limit is also applicable to milk products, which are dried or processed, taking into account the concentration caused by the drying process or by other form of processing (Battacone, 2003; Micheli *et al*., 2005; Rastagi *et al*., 2004). However, the Codex Alimentarius, Joint Expert Committee on Food Additives (JECFA) has established an acceptable level of risk at 0.5 μg kg^{-1} for aflatoxin M1 in fluid milk. The level for AFM1 in milk and dairy products in the United States is tenfold higher (0.50 μg kg^{-1}) than the current level in the EC (FAO/WHO, 2002; Micheli *et al*., 2005). Various countries including Iran have no legal limit for AFM1 in milk and dairy products.

There is a large paucity of data available on occurrence of AFM1 in milk and other milk derivatives in Iran. Thus, in this study, occurrence of AFM1 in raw and pasteurized milk in Urmia city, as one of the largest milk producing regions in Iran, was investigated.

MATERIALS AND METHODS

During the years 2005 and 2006, 72 samples of raw milk were obtained randomly from individual farms in various areas and 72 samples of pasteurized milk were collected in supermarkets in and around Urmia city. Samples were carried to the food chemistry laboratory (Urmia university) within an icebox for detection of aflatoxin M1.

Determination of Aflatoxin M1: Determination of AFM1 was done based on a competitive ELISA using the Immunoscreen AFLA M1 ELISA kit (TECNA S.r.l., Trieste, Italy). Preparation of samples was conducted according to the instructions of the TECNA kit. The kit was stored at 2-8°C and left 2 h at room temperature before use. Samples were refrigerated briefly and then centrifuged at 2-8°C for 10 min at 3000x rpm (Eppendrof, Hamburg, Germany). The upper, creamy layer was removed and aliquot of the lower phase (supernatant without fat) was carefully poured off with a Pasteur pipette. The skimmed milk was used directly in the test.

The AFM1 standards and test samples were added to 96 wells microtiter plate coated with anti-aflatoxin M1 antibodies and incubated for 60 min at room temperature (20-25°C). During incubation, the antibody binding sites were occupied proportionally by the AFM1 concentration. After washing step, conjugate aflatoxin M1-peroxidase was added to the wells and plate was incubated for 30 min at room temperature. The unbound conjugate was removed during the washing. Subsequently, developing solution (Chromogen) was added to the wells and incubated for 30 min. The reaction was ended by adding stop solution. The absorbance measurement was made photometrically at 450 nm. The absorbance values obtained for the standards and samples were divided by the absorbance of the first standard and multiply by 100. Absorbance percentages were taken to the calibration curve performed with standards at different concentration.

RESULTS AND DISCUSSION

A total number of 144 raw and pasteurized milk samples were analyzed with the competitive ELISA kit. The AFM1 detected values are given in Table 1. There was a high incident rate of AFM1 (100%) in both raw and pasteurized milk samples. In Raw milk, thirty one samples (43%) contained aflatoxin M1 at levels of 10-25 ng L^{-1}, fifteen samples (21%) at level 25-50 ng L^{-1} and nine samples (12.5%) at levels more than 50 ng L^{-1}, while 17 (23.5%) contained only traces of aflatoxin (0-10 ng L^{-1}). In pasteurized milk, 53 samples (73.6%) contained low levels (0-10 ng L^{-1}), 18 samples (25%) had levels ranging from 10-25 ng L^{-1} and one sample (1.4%) had levels between 25-50 ng L^{-1} (Fig. 1). None of the samples exceeded the European legislation (50 ng L^{-1}). The mean concentration of AFM1 in raw milk was 24.21 ng L^{-1} (±19.94) while in pasteurized milk it was 8.73 ng L^{-1} (±2.65). Maximum

Table 1: AFM1 contamination of milk samples traded in Urmia, Iran

Sample category	Number	Positive	Average[a] (ng L^{-1})	Range[b] (ng L^{-1})
Raw milk	72	72	24.21	4.3-91.8
Pasteurized milk	72	72	8.73	5.1-28.5
Total	144	144	16.47	4.3-91.8

[a]: Mean of positive samples; [b]: Min-max

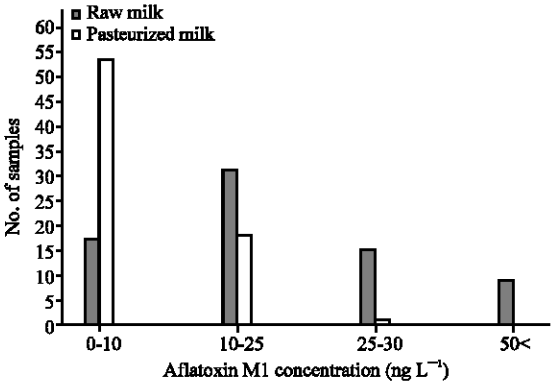

Fig. 1: Levels of aflatoxin M1 in raw and pasteurized milk analyzed

Table 2: The occurrence of AFM1 in milk samples in other countries

Countries	No. of samples		Percent of contaminated milk samples >50 ng L^{-1}	References
	Raw	Pasteurized		
Iran				
Urmia	72		12.5	
Urmia		72	0.0	
Shiraz		624	17.8	Alborzi *et al.* (2006)
Sarab	111		40.0	Kamkar (2005)
Tehran	73		82.2	Karim *et al.* (1998)
Tehran		128	78.0	Oveisi *et al.* (2006)
Greece	81		0.0	Markaki *et al.* (1997)
Turkey		129	47.0	Unusan (2006)
USA		85	64.0	Cathey *et al.* (1994)
India		12	33.0	Rastagi *et al.* (2004)
Turkey		27	3.7	Gurbay *et al.* (2006)
Argentina	56		0.0	Lopez *et al.* (2003)
Argentina		16	0.0	Lopez *et al.* (2003)
Brazil		54	7.4	Oliveira *et al.* (2007)
Italy		316	0.6	Nachtmann *et al.* (2007)
Italy	296		1.7	Decastelh *et al.* (2006)

levels of AFM1 in raw and pasteurized samples were 91.8 and 28.5 ng L^{-1}, respectively. While minimum levels were 4.3 and 5.1 ng L^{-1} (Table 1).

The average AFM1 concentrations in milk in European, Latin American, Far Eastern, Middle Eastern and African diets have been reported by the Joint FAO/WHO Expert Committee on Food Additives to be 23, 22, 360, 5 and 1.8 ng L^{-1}, respectively (Zinedine *et al.*, 2006). In this study, the mean AFM1 level in the total analyzed samples was 16.47 ng L^{-1}. The observed mean AFM1 concentration in this study was nearly 10 times higher than that which was reported in the African diet and lower than those reported for the European, Latin American and Far Eastern diets. In Asia, high incident of AFM1 contamination were found in Indonesia, the Philippines and Thailand (Henry *et al.*, 2001). In this study, the results were in consistent with the study conducted by Zinedine *et al.* (2006). It has been stated, in fact, that the contamination of milk and milk products with AFM1 displayed variations according to geographical region, country and season (Çelik *et al.*, 2005; Panariti, 2001).There are many such studies on the presence of AFM1 in raw and pasteurized milk samples (Table 2).

The results of AFM1 levels, derived in our study form pasteurized samples, were in agreement with Lopez *et al.* (2003). They suggested that the levels of AFM1 in samples of pasteurized milk produced in Argentina were very low and in no case did the levels exceed the recommended limits. Milk samples in Greece had the same contamination as was observed in this study (Markaki and Melissari, 1997). AFM1 level in 40% of positive raw milk samples in Sarab, Iran were higher than the maximum tolerance limit (50 ng L^{-1}) accepted by some European countries (Kamkar, 2005). Karim *et al.* (1998) showed that the incidence of AFM1 in raw milk was 82.2% in Tehran and all contaminated samples had a level of aflatoxin M1 above the European countries standard. However, Alborzi *et al.* (2006) found that 624 (100%) milk samples from Shiraz, Iran were contaminated with AFM1. 17.8% of the samples had AFM1 greater than the maximum tolerance limit accepted by European Union. Taking these information into consideration, our results indicated the lowest level of AFM1 in milk samples in Iran. In the surveys done by FDA laboratory (1995-2000) 185 (4.6%) of 4000 raw milk samples were contaminated with AFM1 at a concentration more than 0.05 μg kg^{-1} and (0.13%) more than 0.5 μg kg^{-1}, which was reported in 1996. Milk samples during the same period, showed AFM1 in 10 cases (4.4 %) of 225 samples containing 0.05-0.5 μg kg^{-1} (Henry *et al.*, 2001). Roussi *et al.* (2002) examined raw and market milk samples for AFM1 contamination, over two periods, in the first sampling, the incident rates of AFM1 contamination in pasteurised milk were 85.4%, where as in the second sampling, 79.6% was reported. They found that none of the pasteurised milk samples exceeded the limit of 50 ng L^{-1}.

Since the aflatoxin contamination is unavoidable, numerous strategies for their detoxification have been proposed. These include physical methods of separation, thermal inactivation, irradiation, solvent extraction, adsorption from solution, microbial inactivation and fermentation. Chemical methods of detoxification are also practiced as a major strategy for effective detoxification. A new approach to the detoxification of aflatoxins is the addition of inorganic sorbent materials, known as chemisorbents; such as hydrated sodium calcium aluminosilicate to the diet of animals. This possesses the ability to tightly bind and immobilize aflatoxins in the gastrointestinal tract of animals, resulting in a major

reduction in aflatoxin bioavailability (FAO/WHO, 2002; Henry *et al*., 2001; Kaniou-Grigoriadou *et al*., 2005).

In this study, 9 samples (6.25%) exceeded the European regulatory limits, most probably because of unusual and considerable using of contaminated feed particularly in traditional and rural animal farms. Due to high contamination of samples, it should be mentioned that feeds for cows in this region might be contaminated with AFB_1. This fact appears to be a serious public health problem at the moment, since all age groups, including infants and children, consume milk worldwide; consequently, it is extremely important to maintain low levels of AFM1 in the feeds of dairy animals. In order to achieve this, dairy cow feeds should be kept away from contamination as much as possible. Since the level of AFM1 was exceeded the maximum limits in 6.25% of the samples assessed in this study, imposing safety limits for AFM1 in milk products in Iran seems to be an urgent need. Hence, there is a need to limit exposure to aflatoxins by imposing regulatory measures on commodities intended for use as food and feed in Iran, as well as in other Asian countries.

CONCLUSIONS

Considering results, it could be concluded that contamination of milk products especially raw milk samples in Urmia is more than European regulation, while the observed mean AFM1 was lower than those proposed for European diets. Contamination of all examined milk samples in our study and its public health importance should be taken into consideration. Accordingly, it is suggested that subsequent studies be conducted on much more milk samples and over more extended periods, in order to obtain data corresponding to various climates, humidity and temperature conditions. Additionally, the government authorities must converse and educate dairy farmers and dairy products consumers with the potential health consequences of aflatoxins.

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