

Antibiotic residues and aflatoxin M1 contamination in milk powder used in Tehran dairy factories, Iran

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Abstract:

BACKGROUND: The presence of aflatoxin M1 (AFM1) and antibiotic residues in milk and milk products is a public health concern. Milk and milk powder have the potential for introducing AFM1 and antibiotic into human diet. In recent years, milk powder has been used on a large scale in dairy factories. Consequently, antibiotic residues and aflatoxin contamination control in these products has gained importance. **OBJECTIVES:** The aim of this survey was to determine the level of β -lactam and tetracycline antibiotic residues and also AFM1 contamination of milk powder used in Tehran dairy factories. **METHODS:** During 12 months (September 2011 to September 2012), 240 samples of milk powder were collected from ten Tehran dairy factories. All samples were analyzed for the presence of AFM1 using ELISA technique. In addition, antibiotic residues were determined by BetaStar Combo test, a rapid assay for both β -lactam and tetracycline antibiotics. **RESULTS:** The samples depicted positive results i.e. 30% and 17.5% for β -lactam and tetracycline antibiotics, respectively. Also, AFM1 was found in 155 cases (64.6%) with an average concentration of 29.85 ± 18.99 ng/L. **CONCLUSIONS:** The results showed the milk powder used by dairy factories is safe in respect of AFM1 contamination and antibiotic residues in Tehran.

Introduction

Antimicrobials are routinely administered to food producing animals for promoting growth and or for therapeutic and prophylactic reasons (Krcmar and Ruzickova, 1996). In the treatment of bovine mastitis, antibiotics are widely used, However, violating the withdrawal time can lead to the contamination of milk at farms. Nowadays, beta-lactams (penicillin G, etc.) and tetracycline (oxytetracycline, etc.) are the most frequently used antibiotics for dairy cows and consequently, the most commonly found antibiotic residues in milk (Gustavsson et al., 2004).

Antibiotic residues are important due to their potential adverse effects on allergic people and high potential antibiotic-resistant microorganisms in humans. Also, the antibiotic residues inhibit the

activity of primitive cultures which produced fermented milk products such as yogurt and cheese. (Jones and Seymour, 1988; Seymour et al., 1988). Veterinary drug residues not only cause potential health risk to human but also change the properties of milk. According to the results of a study by Suhren and Heeschen (1987), there is a high correlation among the presence of tetracyclines and milk pH, the count of somatic cells and the grade of lactation. Therefore, regulatory authorities have enacted maximum residue limits (MRLs) for anti-infective agents in milk. In addition, monitoring programs to control the veterinary drug residues in various animal-origin foods, including milk, are compulsory in most countries (EC Council Directive, 1996). Detectable concentrations of antibiotic residues higher than the MRLs are illegal in milk and dairy products.

Effective monitoring program requires reliable methods for drug residues detection. Various analytical methods have been used to determine antibiotic residues in milk, such as microbiological, chromatographic, immunochemical and enzyme-based tests.

The use of commercial screening tests plays a key role in preventing the unintentional sale and consumption of antibiotic-contaminated milk products. Betastar combo rapid test kit is commonly applied to detect betalactams and tetracyclines residues in milk and milk powder (reconstituted milk) (Kantiani et al., 2009). The detection limits of the kit and MRLs of antibiotics in milk by the EU Commission are presented in Table 1 (Council regulation, 1990).

Aflatoxin M1 is one of the main contaminants of milk and milk products belonging to a group of closely related hepatocarcinogenic bisdihydrofurano metabolites produced by certain species of *Aspergillus*, especially *Aspergillus flavus* (Butler, 1974). AFM1 is the hydroxylated metabolite of Aflatoxin B1 (AFB1) that can be found in milk from livestock which have consumed contaminated feed. About 0.3% to 6.2% of AFB1 in animal feed is converted to AFM1 in milk (Creppy, 2002). AFM1 is relatively stable during pasteurization, sterilization and storage of milk and milk-based products. Even low-concentration intake of AFM1 is a real threat to human health, particularly to children as the main consumers of dairy products. The toxicity of AFM1 was initially classified as Group 2B agent, while it has now moved to Group 1 by International Agency for Research on Cancer (Ghanem and Orfi, 2009). Thus, monitoring AFM1 in dairy products has been conducted and regulatory levels have been established worldwide. Regarding liquid milk, AFM1 levels range from 50 ng/L in the European Union (EU) (Commission regulation, 2006), to 500 ng/L in Codex Alimentarius Commission (CAC, 2010) and United States (US Food and Drug Administration (FDA), 2005).

For these reasons, the control of antibiotic residues as well as the potential AFM1 contamination of milk powder is very important. Most studies on antibiotic residues have been focused on liquid milk but little attention has been paid to milk powder. Given the growing demand for milk products and the persistent threat of antibiotic contamination, the need for the monitoring of powdered milk used in produc-

tion of milk based products for a variety of potentially harmful substances is of the utmost importance. The aim of this survey was to determine the presence of β -lactam and tetracycline residues and AFM1 contamination in milk powder used in dairy factories in Tehran.

Materials and Methods

Sample Collection: In this survey, during 12 months (from September 2011 to September 2012), on the first and fifteenth day of each month, one sample was randomly collected from ten Tehran dairy factories that have the highest milk powder consumption. Samples were transported to the laboratory of Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran and stored at +4°C until analytical tests.

Sample Preparation: All milk powder samples were reconstituted in distilled water (40°C) in the proportion of 1:10 on the day of testing for antibiotic residues detection.

Simultaneously, to determine Aflatoxin M1, 9.1 g of milk powder was dissolved in 100 mL double-distilled water. Next, the solution was heated up to about 50°C and homogenized using a magnetic stirrer. Then 5 mL of reconstitute sample was incubated for 30 minutes at 4°C and centrifuged at 3000 g for 10 minutes. The milk serum was directly used for AFM1 detection with the specific ELISA KIT.

Detection of Antibiotic Residues in Milk Powder by Betastar Combo: The test was carried out according to the manufacturer's instructions (NEOGEN Corporation, USA). It is easily applied and takes approximately 6 minutes.

Determination of AFM1 by Competitive ELISA: The quantitative analysis of AFM1 in samples was performed by competitive ELISA using Ridascreen (R-Biopharm AG, Dermstadt, Germany) according to test kit instructions. 100 μ L of AFM1 standard solutions (1.3 mL each 0, 0.005, 0.01, 0.02, 0.040 and 0.08 μ g/L), and test samples in duplicate were added to the wells of micro-titer plate pre-coated by antibodies against AFM1 and incubated for 60 minutes at room temperature (20-25 °C) in a dark place. Then, the liquid poured out of the wells and the wells were filled with 250 μ L washing buffer and the liquid poured out again. This washing step was

repeated twice. In the next stage, 100 μ L of enzyme conjugate was added to occupy the remaining free binding sites. 250 μ L of washing buffer washed the unbound enzyme conjugates. Then, 50 μ L of enzyme substrate and 50 μ L of chromogen were added to the wells and incubated for 30 minutes at room temperature in a dark place. The reaction was stopped by adding 100 μ L stop solution to each well and absorbance was measured at 450 nm in ELISA reader (Stat Fax 2100, UK). The absorbance values were obtained for the standards and the samples were divided by the absorbance of the first standard (zero standards) and multiplied by 100. Therefore, the zero standards are considered 100% and the absorbance values are expressed in percentage. Detection limit of the kit was 5 ng/L for AFM1.

Statistical Analysis: Statistical analysis was performed using SPSS version 19.0. The data was analyzed by ANOVA and expressed as mean with standard deviation (SD) and also as minimum and maximum concentration of AFM1.

Results

The results of β -lactam and tetracycline residues detection in milk powder are shown in Table 2. In beta star combo assay, 144 samples (60%) were free from both antibiotics and 96 samples (40%) were contaminated by at least one antibiotic. 78 out of 96 positive samples (32%) contained one antibiotic and 18 samples (7%) contained both antibiotics. Meanwhile, 30% and 17.5% of the total samples were positive for β -lactams and tetracycline residues, respectively.

The analytical results of AFM1 levels (ng/L) in milk powder are presented in Table 3. The presence of AFM1 was observed in 155 (64.6%) of all the reconstituted milk samples. 216 samples (90%) were contaminated with less than 50 ng/L. Among positive samples, 24 (representing 15.48%) contained more than 50 ng/AFM1 (EU MRLs). According to Table 4, although the averages of AFM1 concentration obtained from different factories were varied, these differences were not statistically significant ($p > 0.05$).

The highest and lowest contamination levels of AFM1 (72 and 6 ng/L) were found in plant NO. 2 and 3 respectively.

Table 1. Sensitivity of Betastar Combo rapid test kit.

Compound	Minimum detection	Maximum detection	Codex/EU MRL (ppb)
Penicillin G	2	3	4
Ampicillin	2	3	4
Amoxicillin	2	3	4
Oxacillin	3	5	30
Dicloxacillin	3	5	30
Nafcillin	12	14	30
Cephapirin	8	10	10
Ceftiofur	50	100	100
Cephalonium	4	4	20
Cefquinome	-	-	20
Cephalozin	5	7	-
Cephoperazone	5	7	-
Oxytetracycline	60	80	100
Tetracycline	60	80	100
Chlortetracycline	40	50	100
Doxytetracycline	40	50	100

Table 2. Presence of β -lactams and tetracycline residue in Tehran dairy factories.

Factory	Samples tested (n)	Number of positive samples (%)		
		β -lactams	tetracyclines	Both
1	24	8 (33)	5 (20)	2 (8)
2	24	7 (29)	4 (16)	0 (0)
3	24	8 (33)	4 (16)	2 (8)
4	24	8 (33)	5 (20)	3 (12)
5	24	7 (29)	3 (12)	2 (8)
6	24	6 (25)	3 (12)	3 (12)
7	24	6 (25)	5 (20)	1 (4)
8	24	8 (33)	4 (16)	0 (0)
9	24	7 (29)	4 (16)	2 (8)
10	24	7 (29)	5 (20)	3 (12)
Total	240	72 (30)	42 (17)	18 (7)

Discussion

Since the standard of living has risen, people are now paying more attention to the quality of food they consume, including milk products. Therefore, effective control is necessary to ensure the safety of milk and milk products as essential health food. If antimicrobials are misapplied to livestock without monitoring safety recommendations, antibiotic residues can affect milk and milk powder. Nowadays, various screening methods can be used for the detection of antibiotic residues in milk and milk powder in dairy factories. Beta star combo rapid test kit has the capability of detecting the most frequently used antibiotics like β -lactams and tetracyclines at the same time. This method can also be performed rapidly within approximately 6 minutes while the

Table 3. Distribution of AFM1 Contamination in milk powder used in different Tehran dairy factories (ng/L). ^(a) < 5 ng/L AFM1. ^(b) Mean \pm SD of positive samples (containing >5 ng/L AFM1). ^(c) Values in parenthesis indicate % of contaminated samples.

Fatory	Samples tested(n)	Positive samples ^(a) (%) ^(c)	Frequency distribution (n)/AFM1 concentration(ng/L)				Mean	\pm	SD ^(b)
			<5	5-20	21-50	50<			
1	24	15 (62.5)	9	8	3	4	31.80	\pm	23.31
2	24	15 (62.5)	9	5	6	4	38.46	\pm	22.79
3	24	14 (58.3)	10	6	6	2	27.14	\pm	16.05
4	24	15 (62.5)	9	10	5	0	22.06	\pm	11.05
5	24	13 (54.2)	11	7	6	0	22.23	\pm	11.74
6	24	16 (66.7)	8	6	7	3	32.68	\pm	19.75
7	24	18 (75)	6	8	7	3	33.38	\pm	20.98
8	24	15 (62.5)	9	7	6	2	27.86	\pm	18.47
9	24	16 (66.7)	8	8	5	3	31.93	\pm	21.03
10	24	18 (75)	6	9	6	3	28.94	\pm	18.38
Total	240	155 (64.6)	85	74	57	24	29.85	\pm	18.99

Table 4. Occurrence of AFM1 level (ng/L) in milk powder used in Tehran dairy factories ($p>0.05$).

Factory	Samples tested (n)	Minimum	Maximum	Mean	\pm	SD
1	24	13	72	23.24	\pm	20.83
2	24	18	72	25.13	\pm	25.00
3	24	6	58	17.60	\pm	16.54
4	24	14	42	14.58	\pm	13.11
5	24	12	44	13.25	\pm	13.08
6	24	15	72	21.83	\pm	22.37
7	24	12	72	22.55	\pm	25.75
8	24	11	98	19.09	\pm	18.37
9	24	14	72	22.05	\pm	22.20
10	24	18	68	19.57	\pm	22.41
Total	240	-	-	20.11	\pm	20.16

Table 5. Incidence of AFM1 contamination in different types of milk in some region of Iran.

Location	Type of milk	Sample size	Percent of contamination	Percent of contamination on >50 ng/L
Sarab	Raw	111	76.6	40
Shiraz	Pasteurized	624	100	17.8
Tehran	UHT	210	55.2	33.3
Khorasan	Pasteurized	196	100	80.6
Ahvaz	Raw	311	42.1	29.77

Charm test, Delvo test & Copan test require 150 or 180 minutes, three hours and fifteen minutes, respectively (Kantiani, 2009; Zeng et al., 1996).

The results of this survey, which is in line with the findings of a study done by Zvirdauskiene and Salomskiene (2007), indicates that β -star combo test is the best choice for the detection of two main groups of antibiotic residues at the same time because it is the fastest to run, the simplest to use and the easiest to

read.

The results of this survey clearly shows that samples containing β -lactams (30%) are more than those containing tetracyclines (17.5%) and this is similar to the findings of Carlsson and Johnsson (1992) who screened 40,000 milk samples by Delvotest and confirmed the positive samples by Charm test for the presence of β -lactams, tetracycline and aminoglycosides. The results obtained from Charm test revealed that β -lactams were the dominant type of antibiotics found in samples with 51.6 % and a great number of samples were positive for tetracycline. whereas β -lactams were detected in 90-100 % of the samples by Delvotest (Carlsson and Bjorck, 1992). False positive result of screening tests should be noted as an important problem that leads to significant loss for the producers (Zeng et al., 1996). So it is concluded that a combined system of screening method and specific analyses, e.g. HPLC, would be an efficient model for confirmation and verification of false positive samples in dairy factories (Carlsson and Bjorck, 1992).

Furthermore, it is important to employ an accurate, simple and inexpensive method in order to determine AFM1 in milk and milk powder. Enzyme-linked immunosorbent assay (ELISA) is the most common and rapid test to screen the presence of AFM1 in samples. In the present survey, the level of AFM1 in milk powder used in dairy factories was determined by ELISA. 155 out 240 samples (64.6%) were found contaminated by AFM1. AFM1 concentration in all of the reconstituted milk samples was lower than FDA/Codex Alimentarius commission

limit (500ng/L), but it was higher than the maximum tolerance limit accepted by the European Union (50 ng/L) in 24 samples (10 %). The average level of AFM1 in all analyzed samples was 20.11 ± 20.16 ng/L. In addition, the range of AFM1 concentrations was between 22.06 to 38.46 ng/L in positive samples. The reason behind such low levels of AFM1 seems to be the high quality of milk powder imported mostly from reliable sources of European countries which implement strict maximum tolerance level of AFM1 in milk (50 ng/L). Based on the statistical data analysis, there is no difference in the average concentration of AFM1 in milk powder samples obtained from different factories ($p > 0.05$). This shows that the quality of imported milk powder supplied to dairy factories is similar.

Unfortunately, the contamination of powder milk used by dairy factories has not been surveyed in Iran. Most of the studies on AFM1 have been conducted on raw, pasteurized milk as well as the UHT milk by Kamkar, 2005; Alborzi et al., 2006; Heshmati and Milani, 2009; Mohamadi Sani et al., 2010; Rahimi et al., 2010 (shown in Table 5). The variations may be attributed to differences in region, season and, especially, analysis method. Based on the above studies the present situation is not promising and might represent a potential risk for safety and health. Therefore, more must be done to control the presence of antibiotic residues and aflatoxin in milk and milk products.

Based on a study carried out in Brazil (2007), of 12 goat milk powder samples analyzed, 8 (66.7%) tested positive and the mean level observed was 56 ± 0.031 ng/L. Our conclusion is in agreement with recent data and shows high incidence of AFM1 at low concentrations in milk powder (Oliveira and Ferraz, 2007). Ghanem and Orfi (2009) investigated the incidence of contamination of AFM1 in milk powder samples collected from the Syrian market and found that milk powder was almost free from AFM1 contamination with only one sample containing a concentration lower than the European tolerance limit (12 ng/L) (Ghanem and Orfi, 2009), due to the fact that the milk powder was also imported from European sources.

According to studies in European countries, the AFM1 problem is not a serious health threat and does not represent a high risk for public health.

Tsakiris et al. (2013) determined the occurrence of AFM1 in 196 milk samples using ELISA. Only 2 milk samples presented AFM1 levels higher than EU MRL. This is in agreement with our results and the consumers are not exposed to a significant risk from exposure to AFM1 through the consumption of milk in Greece.

Furthermore, Nachtman et al. (2007) set up regional monitoring plan regarding the presence of AFM1 in 316 pasteurized and UHT milk samples in Italy. The results indicated that only 2 samples (one for pasteurized milk and another for UHT milk) showed contaminations higher than 50 ng/L. These results can be associated with applying correct production and storage measured for feed, in order to reduce AFM1 contamination in raw milk.

In the present survey, the mean of AFM1 contamination ($29/85 \pm 18/99$ ng/L) was in agreement with the finding in Portugal. Of 40 pasteurized and UHT milk samples, Eleven featured a contamination above the detection limit (mean $23/4 \pm 24$ ng/L) (Duarte et al., 2013).

Thus, this data indicates that the regulatory authorities should take strict control of milk powder import. The present study shows that β -star rapid and ELISA test should be applied for the detection of antibiotic residuals and for the determination of AFM1 in milk and milk products in dairy factories. In addition, it is concluded that the incidence of AFM1 in consumed milk powder by dairy factories is high, but it has been below the levels that lead to health hazards.

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جستجوی باقیمانده آنتی بیوتیک‌های گروه بتالاکتام و تتراسایکلین و میزان آفلاتوکسین M1 در شیر خشک مصرفی در کارخانجات لبنی استان تهران

نگین نوری* گیتی کریم مهبیاررئیسین حمید خانقاهی ابیانه علیرضا باهنر افشین آخوندزاده بستی فرشته قدمی

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چکیده

زمینه مطالعه: وجود آفلاتوکسین M1 و باقیمانده آنتی بیوتیک‌ها در شیر و فراورده‌های آن از دیدگاه بهداشت عمومی بسیار اهمیت دارد. احتمال ورود آفلاتوکسین و باقیمانده آنتی بیوتیک‌ها به غذای انسان از طریق مصرف شیر و شیر خشک، زیاد است. در سال‌های اخیر استفاده از شیر خشک در کارخانجات لبنی افزایش یافته است. در نتیجه، کنترل وجود باقیمانده آنتی بیوتیک‌ها و آلودگی به آفلاتوکسین M1 در این فراورده‌ها اهمیت یافته است. **هدف:** هدف از این بررسی، تعیین وجود باقیمانده آنتی بیوتیک‌های گروه بتالاکتام و تتراسایکلین‌ها و نیز میزان آلودگی به آفلاتوکسین M1 در شیر خشک مصرف شده در کارخانجات لبنی استان تهران است. **روش کار:** طی ۱۲ ماه (از مهر ۹۰ تا مهر ۹۱) ۲۴۰ نمونه شیر خشک مورد مصرف از ۱۰ کارخانه لبنی استان تهران جمع‌آوری شد. سپس میزان آلودگی هر نمونه به آفلاتوکسین M1 با استفاده از تکنیک الایزا، اندازه‌گیری شد. همچنین باقیمانده آنتی بیوتیک‌ها با استفاده از کیت بتا استارکومبو که یک روش تشخیصی سریع برای آنتی بیوتیک‌های گروه بتالاکتام و تتراسایکلین است، تعیین شد. **نتایج:** ۳۰ و ۱۷/۵٪ نمونه‌ها به ترتیب دارای باقیمانده آنتی بیوتیک‌های گروه بتالاکتام و تتراسایکلین بودند. همچنین آفلاتوکسین M1 در ۱۵۵ (۶۴/۶٪) نمونه آزمایش شده با میانگین غلظت $29/85 \pm 18/99$ ng/L یافت شد. **نتیجه‌گیری نهایی:** نتایج نشان دادند که شیر خشک‌های استفاده شده در کارخانجات لبنی استان تهران، از نظر آلودگی به آفلاتوکسین M1 و باقیمانده آنتی بیوتیک‌ها بی‌خطر هستند.

واژه‌های کلیدی: آفلاتوکسین M1، باقیمانده آنتی بیوتیک‌ها، شیر خشک صنعتی