



Measurement of aflatoxin M1 by ELISA method in milk samples produced by cattle in farms of Shahre Ghods, Shahriar, Iran

Shokofeh Moradi¹, Azar Sabokbar^{2*}, Ramin Hajikhani³, Somayeh Talebi⁴

1. Ms.C of Microbiology, Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran.

2. Professor of Mycology, Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran.

3. Associate Professor of Biology, Department of Biology, Karaj Branch, Islamic Azad University, Karaj, Iran.

4. Ph.D of Microbiology, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

ARTICLE INFO

Article history:

Received 14 March 2017

Accepted 8 May 2017

Available online 1 June 2017

Keywords:

Aflatoxin M1, *Aspergillus*, Cattle farms, ELISA, Milk

ABSTRACT

Aflatoxin M1 (AFM1) is a metabolite of the aflatoxin B1 found in the liver of livestock, as a result of feeding livestock with contaminated food. Certain species of *Aspergillus* are responsible for producing aflatoxin. The present study was performed to evaluate the measurement of AFM1, by ELISA method, in milk samples collected from dairy farms of Shahre Ghods, Shahriar (Tehran, Iran). In this study, during autumn (2016), 82 samples of milk from 41 dairy farms in Shahre Ghods, Shahriar provinces, were randomly selected and assessed AFM1 contamination using the ELISA. On centrifugation of milk, the supernatant including the milk fats was separated and the pellet lacking the milk fat was analyzed through competitive ELISA test, and the amount of aflatoxin was determined. The results obtained from ELISA assay revealed that 90% of samples were contaminated by AFM1 to a measurable amount and only 8 samples (9.7%) crossed the Iran Standard Level of aflatoxin contamination (50 ng/l). Milk and dairy products may be contaminated, and since AFM1 is a serious form of threat for human health and is potentially dangerous, it is essential to constantly assess the livestock feed for aflatoxin contamination to minimize or eliminate its amount in milk or dairy products.

1. Introduction

Mycotoxins, along with other fungal metabolites such as antibiotics, and alkaloids, are mostly produced by fungal cells during the final growth stages of filamentous fungi. These metabolites, known as secondary metabolites, are apparently nonbeneficial for the fungal cells (Sefidgar, 2005; Maktabi, 2011; Hajbar, 2003; Awasthi et al., 2012).

Mycotoxins including aflatoxin, ochratoxin, patulin, fumonisins, zearalenone, and certain trichothecenes such as deoxynivalenol seem to

be of greater importance. Aflatoxins are highly toxic mycotoxins, and the key aflatoxins include G2, G1, B2, and B1 types (Behfar et al., 2012). Aflatoxin B1 (AFB1) is the most harmful natural carcinogen, which is generally produced by the toxigenic strains of *Aspergillus* spp. Aflatoxins are produced as secondary metabolites by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* at temperatures ranging 24-35 °C (Britzi et al., 2013). Aflatoxin production, depends on, social, economic, and the

*Corresponding author: Dr. Azar Sabokbar

Tel: 09125179417

E-mail address: Sabokbar@kia.ac.ir

environmental and climatic (humidity and temperature) conditions, suitable for fungal growth. These toxin-producing fungi can contaminate the food products at various stages of development and production, in particular, during appropriate thermal and moisture conditions. The European Committee for Food of Codex has determined the maximum safe level of aflatoxin in milk and dairy products to be 50 ng/l (Caloni, Cortinovis, Pizzo & d'Azevedo, 2012).

Milk and dairy products form important constituents of the human diet. In terms of nutritional value, they are rich in calcium, phosphorus, required vitamins, and proteins. The proteins found in milk and its products are cheaper and of high biological value compared to other sources of animal and plant proteins. With the development of animal husbandry and milk processing industries, the per capita consumption of milk and dairy products has increased. Aflatoxin produced in the cow's milk that has been fed a contaminated diet, is transmitted to humans and threatens their health (Giovati et al., 2014). Aflatoxin M1 (AFM1) has been also identified in the culture medium of some aflatoxigenic fungi. Studies have shown that *A. flavus* can inhibit the production of aflatoxins group G in *A. parasiticus*. Since the *A. flavus* strain is mainly isolated from contaminated food products, the *A. parasiticus* strain comprises a small percentage of isolates of from the *A. flavus* group. The presence of small amounts of aflatoxin G1 is expected in contaminated samples (Jafarian-Dehkordi & Pourradi, 2013); however, our assumption that *A. flavus* is solely responsible for contaminating all samples that exclusively have AFB1 and AFB2 may not hold true. Finally, one can say that aflatoxin is produced by *A. flavus*, *A. parasiticus*, and *A. nomius* (Gan et al., 2013).

Recently, extensive research is carried out for the development of analytical methods to identify and describe the toxin levels. Timely diagnosis can avoid, irreparable losses to life and economy. This study aimed to measure the AFM1 levels, by ELISA method, in milk samples from dairy farms of Shahre- Ghods, Shahriar (Tehran, Iran).

2. Materials and Methods

2.1. Detection of aflatoxin M1 in milk by competitive ELISA method

To evaluate the aflatoxin level in milk produced in the Shahre- Ghods- Shahriar province, 82 samples were collected from 42 dairy farms at different times during autumn (2016). The samples were stored in sterile containers at -20 °C. The ELISA method was used, which is a competitive enzyme immunologic assay for the antigen-antibody reactions.

2.2. Sample preparation

The samples were removed from the freezer 5 h before the centrifugation time. After thawing the samples, they were transferred using 50 ml pipettes to micro-tubes for centrifugation at 300 RPM (with HERMLE device). As aflatoxins are soluble in water, on centrifugation, the supernatant containing fat was separated. Moreover, the remaining 0.5 cc pellet was transferred to the sterile micro-tubes for testing and was refrigerated until further use.

2.3. ELISA kit assay

The 96-well ELISA kit contains 12 horizontal and 8 vertical bars (Euroclone, Italy). Initially, AFM1, used as a standard, was filled in the first 6 wells of samples (S0, S1, S2, S3, S4, and S5), having aflatoxin concentrations of 0 (control), 5, 10, 25, 50, and 100 ng/l, respectively. The other samples were placed in the remaining wells (82 samples). A volume of 200 µl of distilled water was added into the control well. Moreover, using samplers, a total of 200 µl of the standard samples and other samples were added to the remaining wells. The mixture was gently shaken and incubated for 30 min at room temperature (20-25 °C), preferably away from direct light. At this stage, the wells/micro-wells were washed thrice using a washing buffer with an initial volume of 50 ml (to dilute the buffer, 180 ml of distilled water was added to 20 ml of the buffer), to separate the attached and reactive reagents from the free and unbound reagents. After each washing step, the kit was horizontally placed on a multilayer paper and its contents were removed. Furthermore, 200 µl conjugated enzyme of AFM1 (with a volume of 22 ml a brown lid) was

added to each well, except for the control. The control and the mixture were gently shaken and were incubated for 15 min at room temperature (20-25 °C), preferably away from direct light. Additionally, the wells were washed thrice again. Moreover, using multi-channel microtubes, 200 µl of chromogenic solution (with a volume of 22 µl with a brown lid) was added to each well, including the control well. The addition of chromogen changed the environment color to blue. These microtubes were then incubated for 10 min at room temperature in the dark. To end the reaction, 50 µl of inhibitor solution (sulfuric acid with a white lid) was added to the wells, which changed the environment color from blue to yellow. The mixture was then gently shaken. Finally, the OD of each well was read with the ELISA Reader (Labsystems Multiskan ms) at 450 nm.

2.4. Data analysis

The semi-log standard curve was used to calculate the concentration of aflatoxin in the samples. After reading the ELISA result, the absorption percentage of standards was determined by dividing the absorption standards 2.6 built in the kit with the standard absorption

rate No. 1 (negative control, lacking AFM1) and further multiplying it by 100. Moreover, using the Curve software, a standard curve was drawn, by plotting the logarithm of standard samples' concentrations on X-axis and their absorption percentages at 450 nm on Y-axis; the concentrations of aflatoxin in unknown samples were calculated by applying the dilution factors.

3. Results

The AFM1 contamination values for 90.24%, 8.53%, and 1.23% of the samples were 50 ng/l, 100-200 ng/l, and 200-300 ng/l, respectively (Table 1). Figure 1 shows the standard curve to calculate the concentration of AFM1 in unknown samples.

The milk samples from 41 dairy farms were studied; the samples of 8 farms had AFM1 contamination values ranging 145–208 ng/l, which were above the permitted level of 50 ng/l (Figure 2).

After assessing the animal feed, we observed that the animal feed of 8 dairy farms had been infected with *A. flavus*. Figure 3 shows the range of AFM1 contamination in milk samples.

Table 1. Specifications of aflatoxin M1 ELISA kit standards

Uptake%	Logarithm of the concentration	Concentration in ng/l	Standard
100	0.69	0	S0
69.96	0.753	5	S1
85.50	1	10	S2
67.64	1.39	25	S3
49.30	1.69	50	S4
33.10	2	100	S5

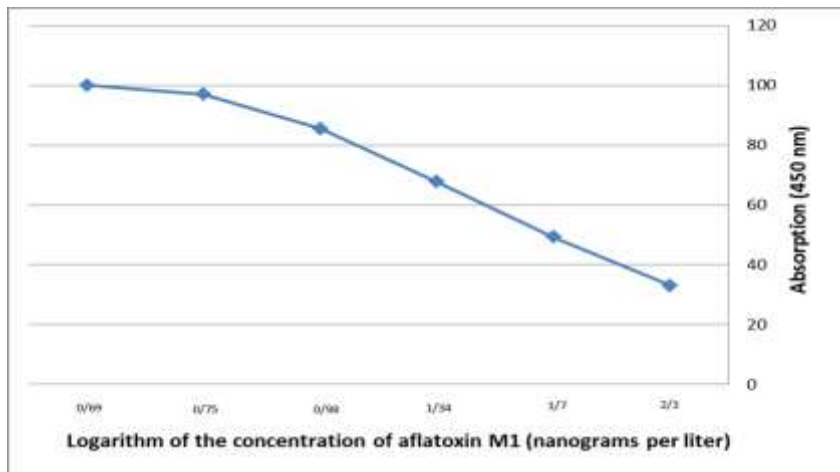


Figure 1. Standard curve to calculate the concentration of aflatoxin M1 in unknown samples

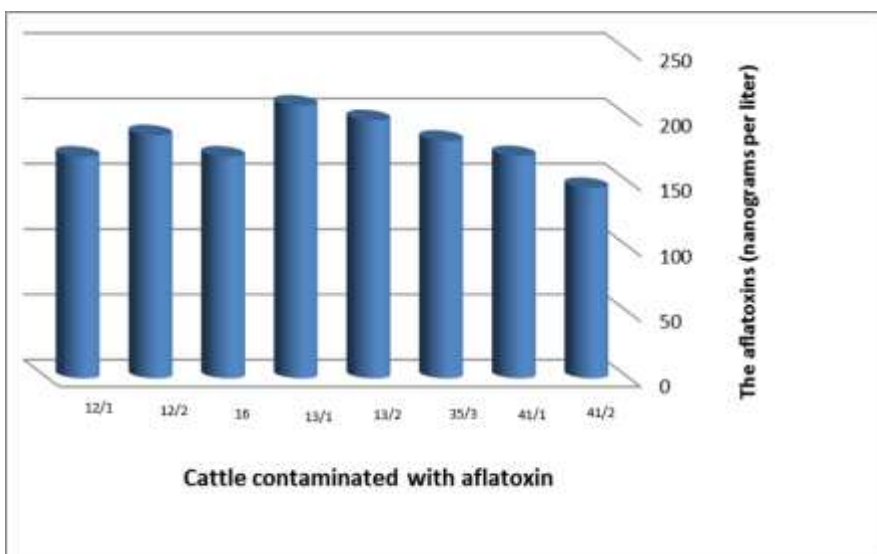


Figure 2. Milk samples from eight dairy farms with higher aflatoxin level than the permitted level

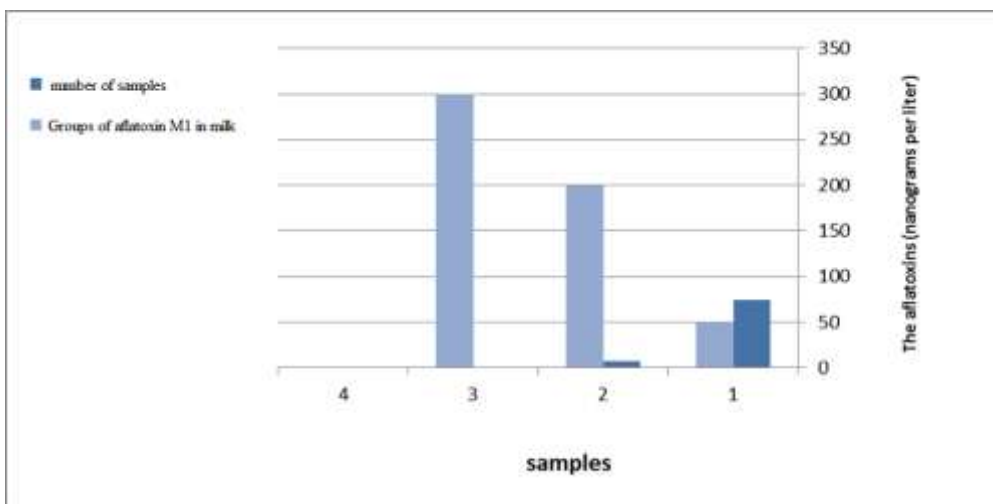


Figure 3. Aflatoxin M1 contamination range in milk samples

4. Discussion

Mycotoxins are the biological materials produced by the toxin-producing fungi in food products, which affect the human health. Therefore, to ensure safety of consumers' health, the existence and amount of different mycotoxins in food products needs to be constantly assessed and in the food chain (Jafarian-Dehkordi & Pourradi, 2013). Many researchers have proven the detrimental effects of aflatoxin in humans, in particular in liver cancer, caused by the consumption of milk and milk products contaminated by AFM1. The livestock feed should be free of AFB1 contamination to improve the quality of milk (Cano-Sancho, et al, 2010; Galvano, 2001).

Previous studies in Iran have shown a high percentage of AFM1 contamination in most milk and dairy products. In a study by Maktabi et al., all the samples were found to be infected by AFM1 (Maktabi, 2011). In another study by Kamyar, 80.7% of the samples had contamination level higher than 50 ng and 26.9% samples showed contamination level above 500 ng/l (Kamyar, 2008). Sefidgar studied 120 samples of raw milk obtained from Babol city, Iran (Sefidgar, 2004), and Ghiasian studied 50 samples of pasteurized milk from Tabriz City, Iran (Ghiasian & Maghsood, 2012); all the samples in both studies were infected with AFM1. Moreover, Hajbar studied 77 samples of pasteurized milk from Sanandaj city, Iran, and reported 17 cases of aflatoxin contamination higher than the permitted level (Hajbar, 2003).

High prevalence of aflatoxin contamination is also seen in other countries. In a study by Galvano, it was found that 78% of milk samples in Italy are infected with AFM1 (Galvano, 2001). Moreover, Polovinski reported that 29.8% of the samples in Serbia have aflatoxin in higher values than the permitted level (Polovinski, 2009). Also, Zinedin reported that 88.8% of the samples in Morocco were contaminated with AFM1 (Zinedin, 2007). In a study by Paraniti, 13% of samples collected in winter and 3% samples collected in the summer showed aflatoxin contamination higher than the permitted levels (Paraniti, 2001). Furthermore, in a study by Sassahara, it was reported that 24% of samples were contaminated with AFM1, and 7% of samples had higher aflatoxin

contamination than the permitted level (Sassahara, 2005).

Our results are consistent with the previous studies and 8 samples in our study had aflatoxin contamination higher than the permitted level, whereas in a study by Hajbar on pasteurized milk, 17 samples were found to be contaminated, which indicates low levels of contamination in samples collected from our study. The results of the present study showed that most of the milk samples obtained from the dairy farms were contaminated with AFM1. The milk samples from the infected cattle corresponded to the feed samples of the farms in terms of *A. flavus* contamination. Thus, the milk samples with aflatoxin contamination lower than the permitted level, reveal that the samples of respective cattle feed are not contaminated by *A. flavus*.

AFB1 can bind to proteins like casein; therefore, it may appear in dairy products. There are inadequate studies in Iran revealing the content of aflatoxin in milk and dairy products. Thus, more research is needed in this regard. In addition, extensive and continuous controls and supervisions must be implemented and guided by the government and relevant ministries. Furthermore, the animal feed should be free of AFB1 in order to produce healthy milk. Due to AFM1 risk for human health, in particular for liver cancer, AFM1 level detection in milk and other dairy products is extremely important (Manetta, et al, 2005; Mohamadi Sani, Khezri & Moradnia, 2012).

Acknowledgments

This study was financially supported by the Islamic Azad University, Karaj, Alborz, Iran.

References

- Awasthi, V., Bahman, S., Thakur, L.K., et al. 2012. Contaminants in milk and impact of heating: an assessment study. *Indian J Public Health*. 56(1):95-99.
- Britzi, M., Friedman, S., Miron, J., et al. 2013. Carry-over of aflatoxin B1 to aflatoxin M1 in high yielding Israeli cows in mid- and late-lactation. *Toxins (Basel)*. 5(1):173-183.
- Caloni, F., Cortinovis, C., Pizzo, F., De Angelis, I. 2012. Transport of aflatoxin M(1) in human intestinal caco-2/TC7 cells. *Front Pharmacol*. 3:111.

- Cano-Sancho, G., Marin, S., Ramos, A.J, et al. 2010. Occurrence of aflatoxin M and exposure assessment in Catalonia (Spain). *Rev Iberoam Micol.* 27(3):130-135.
- Galvano, F. 2001. Survey of the occurrence of aflatoxin m1 in dairy products marketed in Italy 'secondyear of observation. *Food Addit Contam.*18(7): 644-646.
- Gan, N., Zhou, J., Xiong, P., et al. 2013. An ultrasensitive electro chemiluminescent immunoassay for aflatoxin M1 in milk, based on extraction by magnetic graphene and detection by antibody-labeled CdTe quantum dots-carbon nanotubes nanocomposite. *Toxins (Basel).* 5(5): 865-883.
- Ghiasian, S., Maghsood, A. 2012. Infants' exposure to aflatoxin M1 from mother's breast milk in Iran. *Iran J Public Health.* 41(3):119-126.
- Giovati, L., Gallo, A., Masoero, F., et al. 2014. Vaccination of heifers with an aflatoxin improves the reduction of aflatoxin b1 carry over in milk of lactating dairy cows. *PLoS One.* 9(4): e94440.
- Hajbar, M.S. 2003. Determination of aflatoxin M1 in raw milk delivered to milk factory in Sanandaj. *Kordestan Uni J.* 13: 44-50.
- Jafarian-Dehkordi, A., Pourradi, N, 2013. Aflatoxin M1 contamination of human breast milk in isfahan, Iran. *Adv Biomed Res.* 30(2): 86.
- Kamyar, A. 2008. Determination of amount of aflatoxin M1 using ELISA in Industrial milk produced in iran. *Res Develop Aquacul.* 79: 174-180.
- Maktabi, S. 2011. Determination of aflatoxin M1 in milk farms in the region of Ahwaz traditional ELISA. *Veteri J.* 2(7): 44-49.
- Manetta, A.C., Giuseppe, L.D., Giammarco, M. et al. 2005. High performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M1 in milk and cheese. *J Chrom A.* 1083(1-2): 219-2.
- Mohamadi Sani, A., Khezri, M., Moradnia, H. 2012. Determination of aflatoxin m1 in milk by ELISA technique in mashad (northeast of iran). *ISRN Toxicol.* 23:121926.
- Paraniti, F. 2001. Seasonal variations of aflatoxin m1 in the farm milk in Albania. *Arch Hig Rada Toksikol.*52(1): 37-42.
- Polovinski, M. 2009. Two year study of incidence of aflatoxin M1 in milk in the region of Serbia. *Biotech Animal Husban.* 25(5): 713-718.
- Sassahara, M . 2005. Aflatoxin occurrence in foods tuff supplied to dairy cattle and aflatoxin m1 in raw milk in the north of parara state. *Food Chem Toxicol.* 43(6): 981-984.
- Sefidgar, A.A. 2004. Comparison of aflatoxin M1 in milk consumption in winter and summer Babol. *Babol Uni J.* 5(9): 27-31.
- Zinedin, A. 2007. Gonzale-osnayal, sorinojm, moltojc, manesj persence of aflatoxin m1 in pasteurized milk from morocco. *Int j Food Microbial.*114(1): 25-29.