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Survey of aflatoxin residue in feed and milk samples in Kırıkkale province, Turkey*

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Summary: In this study total aflatoxin (AF) and aflatoxin M_1 (AFM_1) contamination in feed and milk samples obtained from the dairy cow farms in Kırıkkale region were studied. A total of 154 dairy cow feed and 154 raw milk samples were obtained from the villages (Delice, Keskin, Sulakyurt, Bahşılı, Yahşihan, Çelebi, Karakeçili, Balışeyh) and central of Kırıkkale province, Turkey, between the years June 2012- August 2013. The quantitative analysis of total AF and AFM_1 in the collected samples were carried out using an enzyme-linked-immunoassay (ELISA) with commercial kits (HELICA biosystems inc.). Total AF was detected in all feed samples and also AFM_1 contamination was found in all of the milk samples. In 5 of 154 feed samples the total AF's were above 20 µg/kg. In none of the milk samples AF was above the legal limit. The mean AF residue level for concentrated feed was 6.43±7.01 µg/kg, and ranged from 0.20 µg/kg to 28.80 µg/kg. The mean AFM_1 residue for milk samples was 1.73±2.20 ng/L, and ranged from 0.08 ng/L to 10.11 ng/L. In conclusion although all of the milk samples were contaminated with AFM_1, the amounts were within the legal limits that are allowed in milk. On the other hand in 3.25% of the feed samples, total AF was above 20 µg/kg. The occurrence of AFM_1 may not be considered as a possible risk for public health. Strategies and monitoring programs to prevent aflatoxin contamination is recommended.

Keywords: AFM_1, dairy cattle feed, ELISA, raw milk, total aflatoxin.

Kırıkkale ilinde süt ve yem örneklerinde aflatoksin kalıntısı araştırılması

Özet: Bu çalışmada Kırıkkale ilindeki sütçü inek işletmelerinden toplanan yem ve süt örneklerinde total aflatoksin (AF) ve aflatoxin M_1 (AFM_1) kontaminasyonuna bakıldı. Türkiye, Kırıkkale İlindeki ilçelerden (Delice, Keskin, Sulakyurt, Bahşılı, Yahşihan, Çelebi, Karakeçili, Balışeyh) ve merkezden Haziran 2012 - Ağustos 2013 tarihleri arasında 154 yem ve 154 süt örneği toplandı. Toplanan numunelerdeki total AF ve AFM_1' in kantitatif analizi, ticari kitlerle (HELICA biosystems inc.) enzim bağlı immünoassay (ELISA) kullanılarak gerçekleştirildi. Tüm yem ve süt örneklerinde total AF ve AFM_1 saptanmıştır. 154 yem örneğinden 5'inde total AF miktarı 20 µg/kg'ın üstünde bulunmuştur. Süt örneklerinin hiçbiri yasal sınırı aşmamıştır. Süt yemlerinde toplam AF düzeyi minumum 0.20 µg/kg, maksimum 28.80 µg/kg olarak tespit edilmiştir ve ortalaması 6.43±7.01 µg/kg'dir. Süt örneklerinde AFM_1 düzeyi minimum 0.08 ng/L, maksimum 10.11 ng/L tespit edilmiştir ve ortalaması 1.73 ±2.20 ng/L'dir. Sonuç olarak, tüm süt örneklerinde AFM_1 kalıntısı bulunsa da bu yasal düzeyin üstünde değildir. Yem örneklerinin ise sadece %3.25 kadarı 20 µg/kg üzerinde total AF içermektedir. Halk sağlığı yönünden AFM_1 bir risk oluşturmamaktadır. Aflatoksin bulaşmalarının önüne geçmek için stratejiler geliştirilmeli ve izleme programları oluşturulmalıdır.

Anahtar sözcükler: AFM_1, çiğ süt, ELISA, sütçü inek yemi, total aflatoksin.

Introduction

Mycotoxins are toxins produced by fungi, which can cause poisoning called mycotoxicosis via contaminated feed and feed ingredients when ingested by animals and humans (21). Aflatoxins are the most important mycotoxins in feeds and feed materials all around the world (39). Toxin produced by *Aspergillus flavus* is called aflatoxin, including 4 significant toxins named Aflatoxin (AF) B_1, B_2, G_1, G_2 (5). There is also AFM_1 found in milk, which is considered to be the metabolite of AFB_1 in dairy animals (37). Approximately %0.8-2.2 of the AFB_1 is excreted as AFM_1 (27). Aflatoxin M_1 is a cytotoxic and genotoxic substance (17), which can be detected in milk 20-24 hours after the ingestion of AFB_1. The conversion of AFB_1 to AFM_1 ends 72 hours after the termination of the intake of AFB_1 by feed (38).

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One of the important steps in milk production is to provide adequate feed and feed stuff in accordance with the requirements of the dairy cows. Animals that are not fed well can easily become ill. The carcass yield and feed consumption have been reduced in animals that receive feed contaminated with aflatoxins. Acute and chronic toxication and presence of aflatoxin residue in the milk are the major concerns in these animals. Cow's milk is one of the most important nutrients consumed by people. Cytotoxic and carcinogenic effects of aflatoxin residues have been scientifically proven. In this regard, the presence of aflatoxin in feed and milk is an important health problem (6, 37). For these reasons, many countries have proposed maximum allowable levels for the aflatoxins. The Turkish Food Codex (TFC) and European Union legal limits for AFM_1 in milk 0.050 µg/kg (36). The permitted values for total AF has not been declared in Turkey yet, but The United States Food and Drug Administration (FDA) has determined a limit of 20 µg/kg for dairy cattle feed (18).

The aim of this study is to detect the total AF and AFM_1 contamination in feed and milk samples obtained from the dairy cow farms in Kırıkkale region.

Materials and Methods

A total of 154 dairy cow feed and 154 raw milk samples were obtained from the 8 towns (Delice, Keskin, Sulakyurt, Bahşılı, Yahşihan, Çelebi, Karakeçili, Balışeyh) and central of Kırıkkale Province, Turkey, from dairy farms, between years June 2012 - August 2013. These feed samples were commercial dairy cow feeds. The feed samples were transported and stored at 4 ^{0}C until analyzed.

There are many methods that can be used to detect AF levels in feed and milk. ELISA method is one of the reliable, cheaper and easier methods that can be used to determine the AF levels (11, 31). The quantitative analysis of total AF in feed, and AFM_1 in the milk samples were done using an enzyme-linked-immunoassay (ELISA) with commercial kits (HELICA Biosystems Inc., HELICA for total aflatoxin-981AFL01LM-96, HELICA for aflatoxin 961AFLM01M-96). Mycotoxin extraction and tests were carried out with respect to the manufacturer's instructions. The milk samples were centrifuged for 10 minutes at 3500 rpm and the upper creamy layer was completely removed, and kept at -20 ^{0}C until analyzed.

The feed samples (20 g each) were added to 100 mL of methanol/water (70:30) and shaken in a blender for 2 min. Then, the suspension was filtered, 5 – 10 mL of the extract passed through a Whatman 1 filter paper. An aliquot of this solution was used in the test.

The milk samples were centrifuged at 3500 g for 10 min at -10 ^{0}C. After centrifugation, the upper cream layer was completely removed and aliquot of the lower phase was carefully poured off with a Pasteur pipette. The skimmed milk (defatted supernatant) was used directly in the test (100 µL).

Two hundred microlitres of the Conjugate was added into each Dilution Well. Later, 100 µL of the standard solutions and prepared samples were added to each well and mixed by priming pipettor at least 3 times. One hundred microlitres of contents from each Dilution Well were transfered to Antibody Coated Microtiter Well and incubated at room temperature for 15 minutes. At the end of incubation, the liquid in the wells was poured out, the Microwell holder was tapped upside down, and an absorbent paper was used to remove the remainder of the liquid. The wells were washed five times with 250 µL of deionized water. After the washing steps, 100 µL of Substrate Reagent was added to the wells and incubated for 5 min at room temperature in the dark. Following that, 100 µL of the stop solution was added to each well and mixed. The absorbance was measured at 450 nm by an ELISA reader (SIRIO S, Italy) against air blank within 15 min. The samples were analyzed according to a computer programme, developed by Helica Biosystems, Inc. The levels of aflatoxin standards used were 0, 1, 2.5, 5, 10 and 20 ng/L. The detection limit of this ELISA method was 1 mg/mL.

Two hundred microlitres of standard solutions and prepared samples in separate wells was added and incubated for 2 h at room temperature in the dark. At the end of incubation, the liquid in the wells was poured out, the Microwell holder was tapped upside down, and an absorbent paper was used to remove the remainder of the liquid. The wells were washed three times with 250 µL of washing buffer. After the washing steps, 100 µL of the conjugate was added to the wells and incubated for 15 min at room temperature in the dark. At the end of incubation, the wells were washed three times with 250 µL of washing buffer. Then, 100 µL of enzyme substrate was added to each well and mixed thoroughly and incubated for 15 min at room temperature in the dark. Following that, 100 µL of the stop solution was added to each well and mixed. The absorbance was measured at 450 nm by an ELISA reader (SIRIO S, Italy) against air blank within 15 min. The samples were analysed according to a computer programme, prepared by Helica Biosystems, Inc. The levels of aflatoxin standards used were 0, 5, 10, 25, 50 and 100 ng/L. The detection limit of this ELISA method was 2 ng/L.

The values were assessed by the program of the relevant company. Quantitative evaluation was performed according to the standard curve, obtained from the computer.

Data were analyzed by "SPSS 15.0 version for Windows". The data were shown as arithmetic means ± standard deviation, also the minimum and maximum values of the data set were given.

Results

In all the collected feed samples, total AF was detected. AFM_1 contamination was found in all of the milk samples. In 5 of 154 feed samples, the total AF was above 20 µg/kg. None of the milk samples were found to be above the legal limit. The mean residue of total AF level for concentrated feed was 6.43±7.01 µg/kg, and ranged from 0.20 µg/kg to 28.80 µg/kg. The mean residue of AFM_1 for milk samples was 1.73±2.20 ng/L, and ranged from 0.08 ng/L to 10.11 ng/L. The results were given in Table 1 and 2.

Table 1. The level of total AF (µg/kg) in dairy cow feed samples collected from Kırıkkale Province.
Tablo 1. Kırıkkale çevresinden toplanan sütçü inek yemlerindeki total AF düzeyleri (µg/kg)

Location	Tested *n*	Positive *n*	Contamination (µg/kg) Range	Mean ±SD	Exceed regulation *n* (%)
Central	19	19	0.30-16.80	4.62±4.96	-
Keskin	15	15	1.20-19.90	8.13±6.53	-
Delice	15	15	0.65-25.30	7.76±9.18	2 (13.33)
Balışeyh	15	15	0.80-28.80	10.02±8.70	1 (6.66)
Karakeçili	19	19	0.70-19.90	5.96±6.78	-
Bahşılı	16	16	0.70-19.90	6.36±7.63	-
Çelebi	19	19	0.50-17.40	6.47±6.32	-
Sulakyurt	17	17	0.30-22.80	3.35±6.52	1 (6.66)
Yahşihan	19	19	0.20-21.80	6.24±6.01	1 (6.66)
Total	154	154	0.20-28.80	6.43±7.01	5 (3.25)

n: number of collected samples,
SD: Standart deviation. There is no legal limit determined for total AF. According to FDA the maximum tolerable limit for total AF is 20 µg/kg (24).
n: Örnek sayısı
SD: Standart sapma. Total AF miktarı için yasal bir limit değeri bulunmamaktadır, FDA'ya göre ise total AF için maksimum tolere edilebilir limit 20 µg/kg' dır (24).

Table 2. The level of AFM_1 (ng/L) in dairy cow milk samples collected from Kırıkkale Province.
Tablo 2. Kırıkkale ve çevresindeki sütçü ineklerden toplanan süt örneklerindeki AFM_1 (ng/L) düzeyleri.

Location	Tested *n*	Positive *n*	Contamination (ng/L) Range	Mean ±SD	Exceed regulation *n* (%)
Central	19	19	0.09-7.79	1.05±2.02	-
Keskin	15	15	0.32-5.79	0.93±1.36	-
Delice	15	15	0.21-6.21	1.40±1.82	-
Balışeyh	15	15	0.26-4.93	1.54±1.42	-
Karakeçili	19	19	0.27-10.11	3.39±3.31	-
Bahşılı	16	16	0.19-9.23	3.82±3.08	-
Çelebi	19	19	0.08-3.70	0.93±0.95	-
Sulakyurt	17	17	0.11-5.11	1.59±1.35	-
Yahşihan	19	19	0.15-3.86	0.98±0.99	-
Total	154	154	0.08-10.11	1.73±2.20	-

n: number of collected samples,
SD: Standart deviation. The Turkish Food Codex (TFC) and European Union legal limits for AFM_1 in milk 50 ng/L (36, 40).
n: Örnek sayısı.
SD: Standart sapma. Türk Gıda Kodeksi Bulaşanlar Yönetmeliği ve Avrupa Birliği'ne göre AFM1 için yasal limit değeri 50 ng/L' dir (36, 40).

Discussion and Conclusion

One of the main source of mycotoxins in dairy cattle breeding is silage hay and straw. And the most common mycotoxins in ruminant rations are aflatoxins (7). Besides having carcinogenic, teratogenic and mutagenic effects, aflatoxins have destructive effects on lipid, carbohydrate and protein metabolism (14). Guthrie (15) observed a decrease in the performance in the lactating cows which received contaminated feed with 120 µg/kg AF. Helferich et al. (16) revealed that 600 µg/kg AF in feed can cause lesion in the liver. It is difficult to diagnose mycotoxin toxicosis as it can cause several health problems like other viral and bacterial infections (40). Therefore, it is important to determine the mycotoxin concentrations in the feed of animals.

The Republic of Turkey's Ministry of Food, Agriculture, and Livestock Announcement No: 2014/11 on undesirable substances in animal feed declared that the maximum limits for AFB_1 are: 20 µg/kg for animal feed and feed products; 5 µg/kg for mixed feed of dairy cattle and calves, and 10 µg/kg for supplementary feeds (25). Doğan and Beyazıt (10) detected 62% AFB_1 residue below 10 µg/kg and 8% AFB_1 residue above 10 µg/kg in 100 feed samples collected from Kars province in Turkey. Şahindokuyucu Kocasarı et al. (34) showed that the most common mycotoxin found in the feed stuff was aflatoxin (60%) in Burdur province, Turkey. Kaya et al. (22) investigated mycotoxin residues in 1200 feed and feed stuff collected from different regions of Turkey by thin layer chromatography 1.08% of these samples were contaminated by mycotoxins. 8-32 µg/kg AFB_1 was detected in 0.5%, 24 µg/kg AFB_2 was detected in 0.08%, 4-40 µg/kg AFG_1 was detected in 0.16% of the samples. In Kars Province, 47.89-66.89 µg/kg total aflatoxin and 27.03-33.25 µg/kg zearalenone were detected in all the analyzed ryegrass silage samples (2).

Oğuz et al. (26) found 1 µg/kg AFB_1 in 3 samples from Konya province and 0.5 ppb AFB_1 in one sample from Mersin province out of 150 collected mixed feed samples. Ekici et al. (11) found total AF and AFB_1 contamination in 72 samples out of 84 feed samples, the levels of these contaminations were found to be within the limits.

There is no legal limit determined for total AF. According to FDA the maximum tolerable limit for total AF is 20 µg/kg (18). In this study 5 (3.25%) of 154 feed samples were found to be above 20 µg/kg for total AF. The mean residue level was 6.43 µg/kg, the highest level was 28.80 µg/kg, whereas the lowest level was 0.20 µg/kg.

There can be AF contamination in milk and milk products as a result of consuming feed and feed stuff contaminated by AF. In fact there are numerous studies showing the occurrence of AFM_1 in the milk and dairy products (8, 9, 13, 19). Humans often exposed to AF from the products obtained from farm animals. The practical importance of this situation is the carcinogenic, teratogenic, mutagenic effects of AF, and the possibility of the inhibition of immune system in humans (14). El Trasi et al. (12) showed that the AFM_1 detected in breast milk can exist because of consuming of raw cow milk contaminated with AFM_1. This shows the importance of determining AFM_1 levels in raw milk. In this study there was a contamination in all of the milk samples, but none of the samples exceeded the legal level (Table 2). These results were in accordance with the results of Oruç et al. (29) which revealed 100% contamination with none of the samples were above the legal limit. In Bursa province 30 raw milk were collected to determine the AFM_1 level by ELISA and the results showed that AFM_1 levels were below the tolerance limits as in our study (30). Oruç and Sonal (28) collected 10 milk samples from street milkmen in Bursa province August, 2001 and found none of the milk samples exceeded the European Union and Turkish tolerance limit.

Buldu et al. (4) found that all of the raw cow milk samples collected from the villages of Kayseri, Turkey were positive for AFM_1 contamination as in our study. Different from our findings, 70% of the samples were found to be above the legal limit in Kayseri.

Şahindokuyucu Kocasarı et al. (35) showed that 91% of the 45 raw milk were contaminated by AFM_1 and 35.5% of these milks were above legal limits of Turkey in Burdur province. Rokhi (32) revealed the contamination of the milk was 65.55% and 31.11% of the milk samples were above the legal limits in Gilan, Iran. They determined the level of AFM_1 as 2.1-131 ng/L. Mohammadian et al. (24), found that the 226 of the 240 raw milk was positive for AFM_1 and 10 % of these were above the legal limits. Again in Ardebil province, Iran, 122 of the collected raw milk were contaminated as in our study. Differently 14.75% of these samples were above legal limits (20). In Greece, between years 1999-2000, 22 (73.3%) of the 30 milk samples were contaminated and 1 (3.3%) of these was above the legal limit (33). Bakırcı (3) detected 12.5-123.6 ng/L AFM_1 in 87.8% of 90 raw milk samples and showed that 44.3% of the samples were above the permitted level 50 ng/L in the Turkish Food Codex in Van province. Akdemir and Altıntaş (1), collected 48 raw milk samples from Ankara region and found 70.8% of 48 raw milk samples were contaminated with 10-817 ng/L of AFM_1, which 33.3% were above the legal limits. Ünüsan (37) investigated the residue level of AFM_1 in UHT milk collected from middle Anatolia, and AFM_1 residue was detected in 58.1% of the milk samples. AFM_1 residue was above the legal limits aproved by European Union in 47% of these contaminated milks. Kireçci et al. (23) reported

51-250 ng/L AFM_1 in 18 of 20 raw milks collected from Sarıkamış, Turkey, which were all above the legal limit 50 ng/L.

Consequently, although all of the milk samples were contaminated with AFM_1, these amounts were determined within the legal limits that are allowed in milk. On the other hand 3.25% of the feed samples were above 20 µg/kg. Based on the results of this study, the occurrence of AFM_1 may not be considered as a possible risk for public health in Kırıkkale. Finally, development of regulatory strategies and monitoring programs to prevent aflatoxin contamination is recommended.

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