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Assessment of Aflatoxin M_1 Levels by Enzyme-linked Immunosorbent Assay in Yoghurt Consumed in Tehran, Iran

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Due to their nutritional value, milk products are popular in Iran. However, aflatoxin M_1 (AFM_1) in milk and dairy products is considered to pose certain hygienic risks for human health. This study was undertaken to determine the occurrence of aflatoxin M_1 in 50 yoghurt samples from 2 dairy factories in summer 2008 and winter 2009. Enzyme-linked immunosorbent assay (ELISA) technique was used for the analysis of yoghurt samples. Aflatoxin M_1 was found 70 % in the yoghurt samples, ranging from 21.1 to 137.6 ng/kg. Toxin level is 17.14 % of the samples exceeded the Iranian national standard limit *i.e.* 50 ng/kg. Also, statistically significant seasonal effect was found for yoghurt samples. The result showed that the contamination of the yoghurt in such a level could be a serious public health problem.

Key Words: Aflatoxin M_1, Yoghurt, Seasonal effect, Enzyme-linked immunosorbent assay, Iran.

INTRODUCTION

Aflatoxins are the best known and most intensively researched mycotoxins produced by different species of *Aspergillus* namely *A. flavus, A. parasiticus, A. bombycis, A. ochraceoroseus, A. nomius* and *A. pseudotamari*, which contaminate agricultural commodities and feeds, particularly at a critical temperature and humidity conditions[1-7]. The main types of aflatoxins are B_1, B_2, G_1, G_2, M_1 and M_2[4]. Aflatoxins are highly toxic, mutagenic, immunosuppressive, teratogenic and carcinogenic compounds that have been implicated to be the causative agents in human hepatic and extrahepatic carcinogenesis[8,9]. Aflatoxin M_1 (AFM_1) has been found in the milk of animals when lactating animals are fed with aflatoxin B_1 (AFB_1) contaminated feeds. The International Agency for Research on Cancer of World Health Organization included aflatoxin B_1 as primary and aflatoxin M_1 as secondary groups of carcinogenic compounds[6,7,10-12]. Aflatoxin M_1 is the monohydroxylated metabolite of aflatoxin B_1 forming in liver by means of microsomial cytochrome P450- associated enzymes[13]. There was a linear relationship between the amount of aflatoxin M_1 in milk and aflatoxin B_1 in the feed, which is consumed by these animals[8,13,14]. Monitoring studies reported that approximately 0.3-6.2 % of aflatoxin B_1 ingested by livestock is transformed to aflatoxin M_1 in milk. However, this transmission rate varies from animal to animal, day to day and one milking process to the next. When the intake of aflatoxin B_1 is stopped, the aflatoxin M_1 concentration in the milk decreases to an undetectable level after 72 h[6,13-19]. Several studies have shown that milk and dairy products are the possible sources of aflatoxin M_1, which could be hazardous for humans, especially infants and children. Thin-layer chromatography, liquid chromatography, high- performance liquid chromatography and enzyme-linked immunoassays are the most current methods for analysis of aflatoxin M_1 in milk and dairy products[3,8,20,21]. Several studies[10,22] indicated that physical treatments such as heat, microwaves, γ- rays, X-rays and ultra-violet light did not affect aflatoxins. Numerous countries have regulated the maximum permissible levels of aflatoxin M_1 in milk and dairy products for human consumption and this condition is different from one country to another. The European commission[23] has established a maximum admissible level of 50 ng/kg for aflatoxin M_1 in liquid milk and dried or processed milk products. However, according to US regulation the level of aflatoxin M_1 in milk should not be higher than 500 ng/kg[24]. According to the Institute of Standards and Industrial Research of Iran[25], the maximum accepted level of aflatoxin M_1 is 50 ng/kg in yoghurt. The purpose of this survey was to determine the contents of aflatoxin M_1 in yoghurt samples produced by two factories that provide Tehran University dairy needs.

TABLE- 1
OCCURRENCE OF AFLATOXIN M1 IN YOGHURT SAMPLES IN WINTER AND SUMMER

Season	Sample tested (n)	Positive samples, *n* (%)	Min-max (ng/kg)	Mean ± SD (ng/kg)	Exceed regulation,[b] *n* (%)
Winter	25	22(88)	21.1-137.6	36. 33 ± 26.85[a]	4(18.19)
Summer	25	13(52)	22-65.1	17.58 ± 20.44[a]	2(15.38)
Total	50	35 (70)	21.1-137.6	26.95 ± 23.64	6 (17.14)

[a]Means ± SD with different letters are significantly different between winter and summer ($P < 0.007$); [b]The ISIRI limit for AFM_1 is 50 ng/kg for yoghurt

EXPERIMENTAL

During winter 2008 and summer 2009, a total of 50 samples of yoghurt produced by two dairy factories were randomly obtained from Tehran University.

Method: The quantitative analysis of aflatoxin M_1 in the samples were based on a competitive enzyme immunoassay by using RIDASCREEN® Aflatoxin M_1 (Art. No: R1101, R-Biopharm, Germany) test kit. Preparation of the yoghurt samples and enzyme-linked immunosorbent assay test procedure were performed according to the method described by R-Biopharm GmbH[26]. Statistical analysis was performed by INSTATA software.

Enzyme-linked immunosorbent assay test procedure: According to the manufacturer's instructions, the diluted samples were used directly in the test. Microtiter wells were used for the standards and the samples were inserted into the microwell holder; then 100 µL of a standard solution and the prepared samples were added to separate wells and incubated for 1 h at room temperature in the dark. The liquid was poured out of the wells and the microwell holder was tapped upside down vigorously (3 times in succession) against absorbent paper to ensure the complete removal of liquid from the wells. All the wells were filled with 250 µL of distilled water and then the liquid was poured out again. The washing procedure was reported once. A 100 µL volume of the enzyme conjugate was added to each well of the used plate and incubated for 1 h at room temperature in the dark. The determination was repeated three times. The samples were incubated for 0.5 h at room temperature in the dark. The free and peroxidase-combined aflatoxins compete for the combining site with antibodies to mouse antibodies immobilized on the plate. Next, the plate was emptied and washed five times with phosphate buffer at pH = 7.2. Then, 50 µL of tetramethylbenzidine and 50 µL of urea peroxide were added and incubated again for 0.5 h in darkness. Following the addition 100 µL of the stop solution to each well, the absorbance was measured photometrically at 450 nm against an air blank and using an enzyme-linked immunosorbent assay reading apparatus[27,28].

RESULTS AND DISCUSSION

Aflatoxin M_1 was found above detectable level in 70 % (35/50) of the yoghurt samples, ranging between 21.1-137.6 ng/kg. Levels of the toxin in 6 (17.14 %) samples exceeded the Iranian national standard limit *i.e.* 50 ng/kg. Regarding seasonal effect influences, the mean concentration of aflatoxin M_1 in yoghurt samples in winter was significantly higher than those obtained in summer (Table-1).

Yoghurt is a famous dairy product in Iran because of its beneficial effect on human health and its nutritive value. Our findings revealed a high level of aflatoxin M_1 in yoghurt samples. This indicates that the milk used in production of yoghurt has been obtained from animals fed with aflatoxin B_1 contaminated feed. In a previous study, Sylos *et al.*[29] reported the absence of aflatoxin M_1 at detectable level in yoghurt samples; but in a recent study, Iha *et al.*[30] and Atasever *et al.*[31] determined aflatoxin M_1 in 72 % of yoghurt samples.

Several researchers[15,18,22,27,29-36] reported the occurrence of aflatoxin M_1 in yoghurt samples, as shown in Table-2. The reported aflatoxin M_1 contamination levels vary from one study to another. It is demonstrated that the milk produced during hot seasons was less contaminated with aflatoxin M_1 than the milk produced during cold ones[37].

TABLE-2
INCIDENCE AND LEVELS OF AFLATOXIN M1 IN VARIOUS YOGHURT REPORTED IN PREVIOUS STUDIES

Country	Year	No. of samples	Positive (%)[a]	Range (ng/kg)
Brazil (29)	1996	30	0	ND
Italy (22)	1998	114	79.8	1-496.47
Korea (32)	2000	60	51.7	17-124
Italy (33)	2001	120	60.8	1-32.1
Kuwait (34)	2001	5	20	10-210
Taiwan (15)	2004	24	12.5	7-44
Portugal (18)	2004	96	18.8	19-98
Turkey (27)	2006	177	58.75	1-150
Turkey (35)	2006	40	80	62-366
Iran (36)	2010	68	66.1	15-119
Brazil (30)	2011	53	72	10-529
Turkey (31)	2011	80	87.5	10-475

ND: Not determined; [a]Indicates percentage of total samples

Conclusion

Results of this studyimply that more emphasis should be given to the routine inspection of aflatoxin M_1 present in milk and dairy products in Iran. Also, the governmental agencies should inform farmers and dairy factories about the health consequences of aflatoxins. Due to the fact that all the age groups specially infants consume milk and dairy products, it is important to inspect and control of the products regularly.

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