Occurrence Of Aflatoxin M_1 In Milk Collected From Kafr El-Sheikh Governorate , Egypt

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ABSTRACT

Ninety samples of raw, ultra high temperature (UHT) and flavoured UHT milk (30 samples each) were obtained from supermarkets in Kafr El-Sheikh, Egypt. The occurrence and concentration range of Aflatoxin M_1 (AFM_1) in the samples were investigated by competitive enzyme-linked immunoabsorbent assay (ELISA) method. AFM_1 was found in 63 (70%) out of 90 milk samples examined. The levels of AFM_1 in 16 (25.4%) samples were higher than the maximum tolerance limit (50 ng/l) accepted by some European countries while none of the samples exceeded the prescribed limit of US regulations (500 ng/l). The highest mean concentration of AFM_1 was recorded in raw milk samples (55.7 ± 6.7ng/l). The lowest mean concentration of AFM_1 was recorded in flavoured UHT milk samples (18.8± 4.8 ng/l). While, the mean concentration in UHT milk samples was 23.1± 4.7 ng/l. It was therefore concluded that, the levels of AFM_1 in milk especially raw samples consumed in Kafr El-Sheikh, Egypt were high and seemed to pose a threat to public health.

INTRODUCTION

Mycotoxins are large group of compounds, secondary metabolites of fungi, which can contaminate broad number of feed and food. Aflatoxins are highly toxic secondary metabolites produced by several Aspergillus species that can be found in cows milk. Aflatoxin M_1 (AFM_1), the major metabolite of aflatoxin B_1 (AFB_1) is classified by the international Agency of Research on Cancer as class 2B, possible human carcinogen *(1)*, has now moved to Group1 *(2, 3)*. Hence, the detection and determination of this mycotoxin in foods particularly in dairy products is one of the increasing interests *(4)*.

Presence of mycotoxins in dairy products reflects the contamination of feedstuffs. AFB_1 is poorly degraded by rumen microorganisms *(5)*. Absorbed AFB_1 is principally metabolized in the liver into AFM_1, a metabolite as toxic as the parent toxin, which appears in milk. The amount of AFM_1 found in milk represents normally 1 to 2% of the ingested AFB_1. However, it can be as high as 6% in high-producing cows *(6)*.

Although AFM_1, the hydroxylated metabolite of AFB_1 is less carcinogenic and mutagenic than AFB_1, it exhibits a high level of genotoxic activity and certainly represents a health risk hazard because of its possible accumulation and linkage to DNA. Monitoring of AFM_1 levels in animal studies has shown that the rate between the amount of AFB_1 ingested by cows and the quantity excreted in milk is usually 0.2 to 4% *(7)*.

According to Stoloff *(8)*, milk has the greatest demonstrated potential for introducing AF residues from edible animal tissues into the human diet, and taking into account that pasteurization process and even those using UHT, Ultra High Temperature, techniques do not affect AFM_1 concentration because of its heat stability *(9)*. Moreover, as milk is the main nutrient for growing young, whose vulnerability is noteworthy and potentially more sensitive than that of adults, the occurrence of AFM_1 in human breast milk, commercially available milk, and milk products is one of the most serious problems of food hygiene. For this reason, many countries have regulations to control the levels of AFB_1 in feeds and to propose the maximum permissible levels of AFM_1 in milk to reduce this risk *(10)*.

Regulatory limits for AFM_1 throughout the world are highly variable, depending on the

degree of development and economic involvement of countries and may vary from one country to another *(11)*. The European Community and Codex Alimentarius prescribe that the maximum level of AFM_1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg *(12)*. However, according to US regulations the level of AFM_1 in milk should not be higher than 500 ng/kg *(13)*. In Austria and Switzerland, the maximum level is further reduced to 10 ng/kg for infant food commodities *(14)*. Thus, there are differences in maximum permissible limit of AFM_1 in various countries *(15)*.

Many analytical and immunological methods such as TLC, HPLC and ELISA are available for estimation of AFM_1 in milk. With the availability of monoclonal and polyclonal antibodies against aflatoxins, various simple sensitive and specific ELISA tests have been developed for aflatoxin analysis *(16)*. ELISA method is a quick, reliable and cost effective for estimation of AFM_1 and has been included in the official collection of test procedures by the German Federal Board of Health *(17)*.

The production and consumption of ultra high temperature treated (UHT) milk and flavoured UHT milk have been increased in Egypt. There is no enough information about the occurrence of AFM_1 in UHT milk in Egypt. For this purpose, the present investigation was designed to determine the presence and level of AFM_1 in UHT milk and flavoured UHT milk samples in addition to raw milk samples that especially sold and consumed in Kafr El-Sheikh Governorate, Egypt, and to compare the obtained results with maximum AFM_1 tolerance limits of (50 ng/l) in milk that accepted by European Legislation 466/2001/EC *(12)*.

MATERIALS AND METHODS

1. Samples

Ninety samples of raw, UHT milk and flavoured UHT milk (30 samples each) were brought from different supermarkets in kafr El-Sheikh Governorate, Egypt. All samples were analysed before their expiry date.

2.Method

Quantitative analysis of AFM1 was carried out using an Enzyme Linked Immunoassay (ELISA) commercial kit (RIDASCREEN®, Darmstadt, Germany) according to the instructions of manufacturer.

3. Reagents

Most of the reagents used were contained in the RIDASCREEN test kit. AFM_1 standard solutions used for the construction of the calibration curve were at levels of 0 (zero standard), 5 ppt, 10 ppt, 20 ppt, 40 ppt, 80 ppt, all included in the ELISA test kit.

4. Preparation of samples

Ten milliliter of the milk samples were chilled to 10 C° and centrifuged for 10 min at 3500g (8000 rpm). The upper oily phase was completely collected. An aliquot (100 µl/ well) of the lower oil-free phase was used in the test.

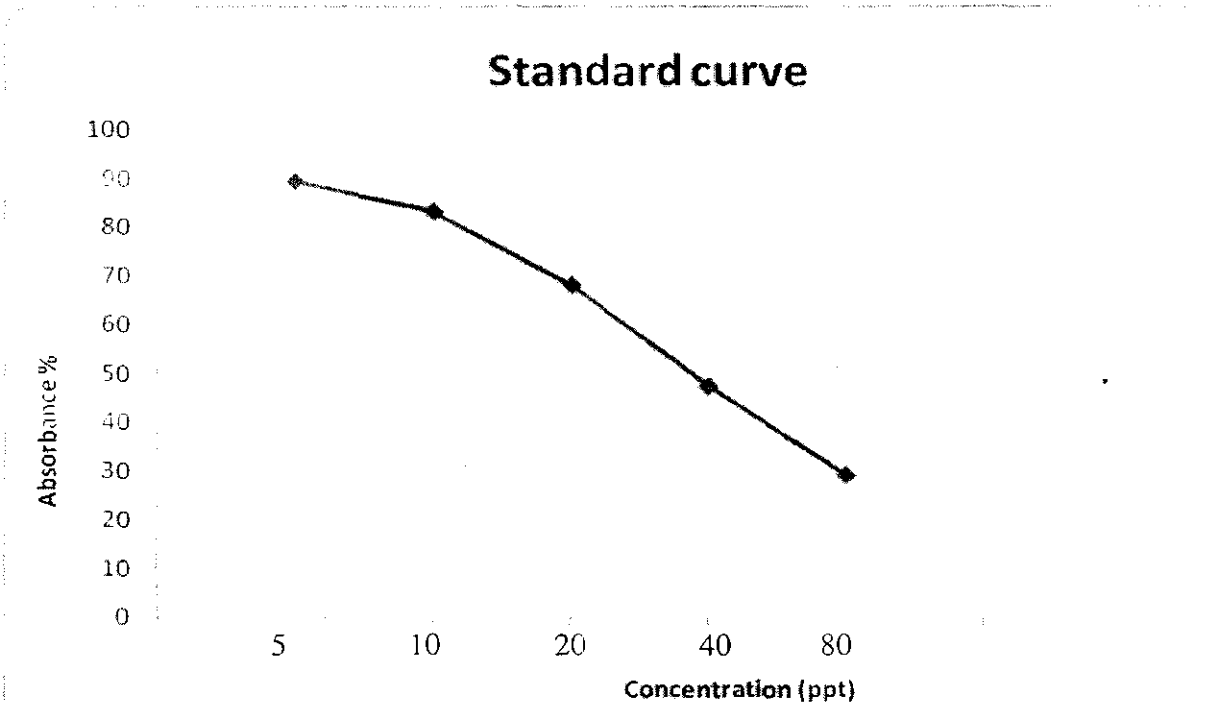
5. Test procedure

According to the manufacturer's instructions, a sufficient number of micro titer wells were inserted into the micro well holder for all standards and samples. 100 µl standard solution and prepared samples in separate well were added and mixed gently by shaking the plate manually and incubated for 30 min at room temperature in the dark. At the end of incubation, the liquid in the wells was poured out, and the micro well holder was tapped upside down on an absorbent paper to remove the remainder of the liquid. The wells were washed three times with 250 µl washing buffer. 100µl of the enzyme conjugate (peroxidase conjugated AFM_1) was added to each well and mixed gently by shaking the plate manually and incubated 15 min at room temperature in the dark. At the end of incubation, the liquid in the wells was poured out. The wells washed three times with 250µl washing buffer. 100µl substrate/chromogen were added to each well and incubated for 15 min at room temperature in the dark. Following the addition 100µl of the stop solution to each well, the absorbance was measured photometrically at 450 nm against an air blank.

6. Evaluation of AFM_1

The mean of the absorbance values obtained for the standards and the samples were divided

by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus made equal to 100% and the absorbance values are quoted in percentages. The absorption is inversely proportional to the AFM_1 concentration in the sample. The calibration curve was virtually linear in the 10 – 80 ppt range. According to the test preparation record, the lower detection limit is 5 ppt for milk. Also according to the instructions for use of the RIDASCREEN kit, the recovery rate in spiked milk (10–80 ppt range) is 95% with a mean coefficient of variation of 14%.



7- Statistical analyses

The obtained results were statistically evaluated according to Rosner *(18)*.

RESULTS

In this study, 90 samples of raw, UHT and flavoured UHT milk (30 samples each) were analyzed to evaluate the concentration of AFM_1. The results revealed that 14 samples (15.6%) had AFM_1 below the detection limit (5 ppt), while AFM_1 was found in 18 (60%), 21 (70%), 24 (80%) of examined raw, UHT and flavoured UHT milk samples respectively with total percentage of 70% (63 samples). The range of contamination levels varied among different milk types. The highest mean concentration of AFM_1 was found in raw milk samples (55.7± 6.7 ng/l), while the lowest (18.8± 4.8 ng/l) was found in flavoured UHT milk samples. Concerning to UHT milk samples the mean concentration of AFM_1 was 23.1± 4.7 ng/l (Table 1).

The frequency distribution of examined milk samples based on their AFM_1 concentration was shown in Table 2. Forty seven (74.6%) samples (7, 18 and 22 of raw, UHT and flavoured UHT milk respectively) had AFM_1 concentration within the range of 5–50 ng/l. While sixteen (25.4%) samples (11, 3 and 2 of raw, UHT and flavoured UHT milk respectively) contained AFM_1 >50 ng/l.

Table 1. Concentration of Aflatoxin M_1 (ppt) in the examined milk samples

Samples	No. of examined samples	Positive samples		ND* samples		Concentration ng/l (ppt)		
		No.	%	No.	%	Min.	Max.	Mean± SE
Raw milk	30	18	60.0	2	6.7	11	102.5	55.7± 6.7
UHT milk	30	21	70.0	7	23.3	6	85	23.1± 4.7
Flavoured UHT milk	30	24	80.0	5	16.7	5	94	18.8 ± 4.8
Total	90	63	70.0	14	15.6	5	102.5	30.8 ± 3.8

ND*: not detected (below the detection limit (5 ppt).

Table 2. Frequency distribution of examined milk samples based on their Aflatoxin M_1 concentration.

Concentration ng/l (ppt)	Raw milk		UHT milk		Flavoured UHT milk		Total	
	No.	%	No.	%	No.	%	No.	%
5 – 50	7	38.9	18	85.7	22	91.7	47	74.6
> 50	11	61.1	3	14.3	2	8.3	16	25.4
Total	18	100.0	21	100.0	24	100.0	63	100.0

DISCUSSION

Since, milk is a major commodity for introducing aflatoxins in human diet, and several investigators *(8, 9)* have showed evidence of hazardous human exposure to AFM_1 through dairy products, many countries carried out studies about the incidence of AFM_1 in milk.

In this study, raw milk showed the highest mean of contamination, in addition, eleven (61.1%) samples of which showed AFM_1 levels higher than the maximum tolerance limit (50 ng/l) *(12)*. This may be attributed to the absence of the AFM_1 monitoring protocol in dairy farms but in UHT and flavoured UHT manufactures; there may be some restrictions for incoming milk.

Our study confirmed the incidence and the high contamination level of AFM1 in milk produced in Egypt, as shown in a previous study in which three of 15 cows' milk samples were found positive for AFM_1 with mean value 6.3 ppb. *(19)*. High incidence of AFM_1 contamination is attributed to the extensive use of cereals in dairy cattle farms beside the favourite temperature and humidity for fungal growth in Egypt. Mycotoxins in milk are indicators of feed contamination (e.g. AFM_1 is a marker for AFB_1 in feeds and appears in milk within 12 h post-ingestion) *(20)*. Contamination of animal feed with aflatoxins was studied, where a total of 1503 of commercially mixed feeds, cereal grains, milk replacers, protein concentrates and processed animal feeds were collected during the years 1991-1994 from commercial mills and animal feeding stores located throughout Egypt *(21)*. Aflatoxins were detected in 619 (41 %) samples in the range of 1-2000 ppb. The commercially mixed feeds were found to be more contaminated with aflatoxins than were in the cereal grains.

Furthermore, high incidence similar to our results was reported in North Africa. Forty-nine samples of raw cow's milk were collected directly from 20 dairy factories in the north-west of Libya and analysed for the presence of AFM_1. Thirty-five milk samples (71.4%) showed AFM_1 levels between 0.03 and 3.13 ng ml^{-1} milk *(22)*. Also, AFM_1 was detected in Wad Medani, Sudan , in 3 out of 5 (60%) bulk milk samples with an average concentration of 160 ng/l *(23)*.

In Asia, high incidences and levels of aflatoxin M_1 contamination were found. For example, in Thailand, out of 310 liquid milk

samples, 261 (>84%) were contaminated with AFM_1 with concentrations of >0.05 µg/kg, and 58 samples (19%) contained AFM_1 >0.5 µg/kg, with a maximum of 6.6 µg/kg *(24)*. In Portugal and Spain, the incidence rate of AFM1 contamination above maximum level was 2.87%, and 3.3%, respectively *(25, 26)*. It has been indicated that many countries in Europe showed relatively low levels of contamination of AFM_1 in milk samples because of a result of stringent regulation of AFB_1 in dairy cattle feed *(27)*.

Concerning UHT milk, the using of Ultra High Temperature techniques, do not affect AFM_1 concentration because of its heat stability *(9)*. In Iran *(28)*, lower incidence of AFM_1 (55.2%) than that estimated in our study (70%) was reported. However, UHT samples that have AFM_1 levels higher than the maximum tolerance limit were 33.3% but were 14.4% in our results. In addition, AFM_1 incidence in UHT milk samples that were produced by different plants in province of Tehran was 100%. The range of contamination levels varied from 19.40 to 93.60 ng/kg, while the mean value was 65.50 ng /kg. Almost 79.92% of the contaminated samples exceeded the maximum acceptable level (50 ng/kg) *(29)*.

Studies done in Spain and Pakistan, reported the incidence rate of AFM1 in UHT milk samples was 29.8% and 11.3% whereas 4.26% and 7.59% of contaminated samples exceeded legal limit (0.05 µg/l), respectively *(30)*. In Portugal and Greece, the incidence rate of AFM_1 in UHT milk samples was 84.2% and 82.3%, respectively, that 2.86% of samples in Portugal and none of them in Greek contaminated exceeding legal limit *(31)* .

In conclusion, the levels of AFM_1 in milk samples produced and consumed in Egypt especially Kafr El-Sheikh Governorate are high and seem to pose a threat to public health. The result of this study and some previous studies about contamination of dairy products with AFM_1 imply that more emphasis should be given to the routine AFM_1 inspection of milk and dairy products as well as storage of animal feed in Egypt.

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الملخص العربي

مدى تواجد الافلاتوكسين (م١) في الالبان المجمع من محافظة كفر الشيخ

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أجريت هذه الدراسه في محافظة كفر الشيخ , بجمهورية مصر العربيه على تسعين عينه من اللبن الخام , اللبن المعقم و مثيله المنكه (ثلاثون عينة من كل نوع) تم تجميعها من محال البقالة بالمحافظة. و قد تم الكشف عن وجود الافلاتوكسين م١ فى عينات اللبن با ستخدام اختبار الاليزا، ولقد تبين من الدراسة وجود الافلاتوكسين م١ في١٥,٦% من العينات بكمية أقل من الحد الأدنى للكشف عنها (٥ نانوجرام/لتر)،بينما تبين وجودها فى سبعين بالمائة من العدد الكلي للعينات بتركيزات أعلى من الحد الأدنى للكشف عنها بالاضافه إلي وجودها بمعدلات تفوق المسموح به عالميا في٢٥,٤% من العينات. و قد أوضحت الدراسة أيضا ان اعلي متوسط تركيز للافلاتوكسين م١ (٥٥,٧ ± ٦,٧ نانوجرام/لتر) وجد فى اللبن الخام, أما بالنسبة لعينات اللبن المعقم و المعقم المنكهه فقد كان متوسط التركيز ٢٣,١± ٤,٧ و١٨,٨± ٤,٨ نانوجرام/لتر علي التوالي. و من ثم فان معدل وجود و مستوي تركيز الافلاتوكسين م١ في الالبان المستهلكه في مصر و خاصة محافظة كفر الشيخ عاليه و تمثل خطرا علي الصحه العامه.