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A Study on the Occurrence of Aflatoxin M_1 in Raw and Sterilized Milk in Eljabal Alakder Region of Libya

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ABSTRACT

AFM1 concentration of 22 raw milk samples collected from farms in different areas around El-baida city, Eljabel Alkhader province, Libya and 42 sterilized milk samples were analyzed. The method used was ELISA technique. Results showed that in one raw milk sample (4.5%) the AFM_1 concentration was less than 5 ng/L and none of contaminated raw milk samples exceeded the Libyan regulation limits of 50ng/L for liquid milk. For the sterilized milk samples the range of AFM_1 was below the detection limit to 160 ng/L with 9 (21%) samples above Libyan legal limit.

INTRODUCTION

Milk and dairy products are fundamental components in the human diet and may be the principle way for entrance of aflatoxin into the human diet body (1).

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated as causative agent in human hepatic and extrahepatic carcinogenesis (2, 3).

Aflatoxins are secondary metabolites produced by species of Aspergillus, especially *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin M_1 (AFM_1) may be found in the milk of animals that are fed with aflatoxin B_1 (AFB_1) containing feed (4,5). There was a linear relationship between the amount of AFM_1 in milk and AFB_1 in feed consumed by the animals (6).

International agency for research on cancer (IARC) of WHO included AFB_1 as primary and AFM_1 as secondary groups of carcinogenic compounds (6, 7).

Many countries have carried out various control and inspection programs on this subject fairly concerning about public health for many years. According to the results obtained, maximum aflatoxin levels were determined for food and feed.

Regulatory limits throughout the world are influenced by considering each country's conditions and may vary from one country to another (8, 9, 10). The European Community and Codex Alimentarius prescribe that the maximum level of AFM1 in liquid milk and dried or processed milk products should not exceed 50ng/kg (11). However, according to US regulations, the level of AFM_1 in milk should not be higher than 500ng/kg (12). In Austria and Switzerland, the maximum levels are further reduced to 10ng/kg for infant food commodities (13).

Since milk is a major commodity for introducing aflatoxins in human diet, and evidence of hazardous human exposure to AFM_1 through dairy products has been shown by several investigators (14). Many countries have carried out studies about the incidence of AFM_1 in milk. In most of them, samples have been found whist exceed the limit imposed by many countries of 50 ng/L.

The purpose of this survey was to determine natural occurrence and levels of AFM_1 in raw milk produced in Eljabal Alakder region also in sterilized milk and to compare the obtained

results with maximum AFM1 tolerance limits (50 ng/l) accepted by Libyan standards.

MATERIALS AND METHODS

Sampling:-

A total of around sixty six samples were collected from the north, south, east and west of Elbaida city in Eljabal Alakder state - Libya, during 2015. The samples composed of raw milk 23 samples. Collected raw milk transported to the laboratory in refrigerated containers (+4°C) and stored at -20°C until analysis.

Local and imported sterilized cow milk 43 samples were bought from different supermarkets in Elbaida city, all samples were analyzed before expiry date.

Method:-

Quantitative analysis of AFM_1 was carried out using an Enzyme Linked Immunoassay (ELISA) commercial kit (RIDASCREEN, Darmstadt, Germany)

Reagents

Most of the reagents used were contained in the RIDASCREEN test kit, standard solutions used for the construction of the calibration curve were at levels of 5.0, 10.0, 20.0, 40.0 and 80.0 ng/l all included in the ELISA test kit.

Sample Preparation:-

For raw milk and sterilized cow milk, 10 ml were chilled to 10^0C and centrifuged for 10 min at 3500g. The milk serum below the fat layer was sampled and directly assay for AFM1 using a specific ELISA Kit.

Test procedure:-

According to the manufacturer's instructions, a sufficient number of microtiter wells were inserted into the microwell holder for all standards and samples to be run in duplicate. 100 µl standard solution and prepared samples in separate duplicate wells 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 microwell holder was tapped upside down on an absorbent paper to ensure complete removal of liquid

from the wells. The wells were washed twice with 250ul washing buffer. 100µl of the enzyme conjugated (peroxidase conjugated AFM_1) was added to each well and mixed gently by shaking the plate manually and incubated 15 min at room temperature (20-25^{0}C) in the dark. At the end of incubation, the liquid in the wells was poured out. The wells washed three times with 250ul washing buffer. 100µl substrate /chromogen (brown cap) were added to each well and incubated for 15 min at room temperature in the dark. Following the addition, 100µl of the stop solution (yellow cap) to each well and mixed gently by shaking the plate manually and the absorbance was measured at 450 nm against an air blank within 15 min after addition of stop solution.

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 samples. The calibration curve was virtually linear in the 5.0 – 80 ng/L range.

$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 = \%\ \text{absorbance}$$

According to the test preparation record, the lower detection limit is 5 ng/L for milk samples.

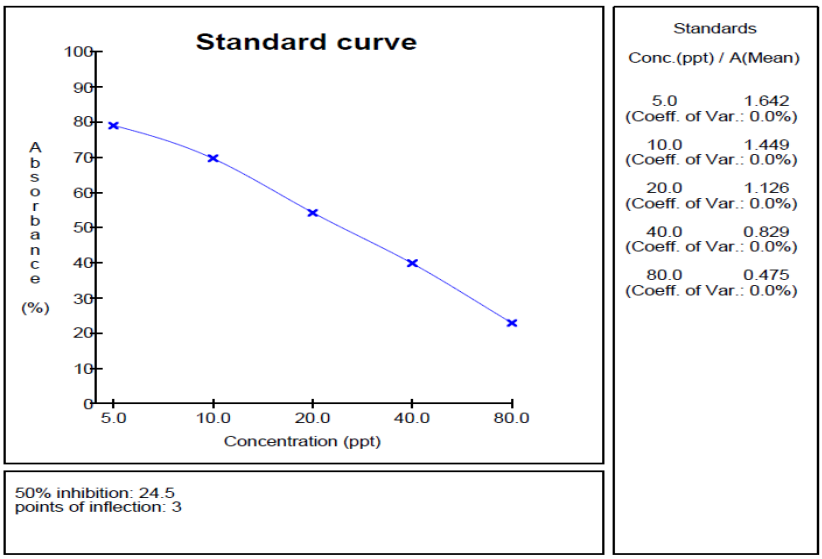

Fig. 1. Calibration curve of Aflatoxin M_1 by competitive ELISA at 450nm

RESULTS

The percentages of absorbance obtained in the competitive enzyme immunoassay with the calibration curve (Fig. 1) allows calculate the AFM_1 concentration in µg/L in the positive samples (Table 1,2) for each kind of milk raw milk, local and imported sterilized milk and the corresponding mean value.

The occurrence and the distribution of AFM_1 concentration in raw milk samples obtained from farms in different areas around El-baida city–El Jebel Alkhader province are presented in Tables 1. of the 22 raw milk samples analyzed, 95% were found to be

Table 1: - Levels of Aflatoxin M1 in raw milk:-

Regions	Sample analyzed	Positive samples	AFM1 concentration (ng/L) range	means	Exceed legal limit (%)
West region	**6**	**5(83%)**	**N.D – 8.91**	**6.73**	**0.0**
East region	**6**	**6(100%)**	**5.19 – 10.93**	**7.48**	**0.0**
North region	**3**	**3(100%)**	**6.07– 9.11**	**7.27**	**0.0**
South region	**7**	**7(100%)**	**5.66 –10.17**	**7.47**	**0.0**
Total	**22**	**21(95%)**	**N.D – 10.93**	**7.24**	**0.0**

Libyan and EC limits is 50 ng/L

N.D= not detected

Contaminated with AFM_1. The incidence of AFM_1 in milk from West was 83%, while from East, North and South regions the contaminations were 100%. The overall mean level of AFM_1 in the samples was 7.24 ng/l. However, none of the samples was higher than the maximum tolerance level of AFM_1 in liquid milk regarding Libyan standard, and then the maximum tolerance limit accepted by European Union (EU) and Codex Alimentarius Commission (50 ng/L), even for infant consumption (15).

The results of the analyses of Aflatoxin M_1 (AFM_1) level (ng/L) in local and imported sterilized milk are shown in Table 2.

Out of the 42 sterilized milk samples analyzed, 30 (71%) samples were imported and the rest 12 (29%) were locally produced. The contamination in the local sterilized milk samples ranged from below the detection limit to 160 ng/L, with 8 of the 12 (66.6%) locally produced sterilized milk samples exceeding the Libyan and EC limit for AFM_1 of 50 ng/kg. The sterilized milk imported samples ranged from below the detection limit to 160 ng/L and only six samples (20%) was contaminated at a level above the Libyan limit and the European regulatory maximum levels, on the other hand, aflatoxin M_1 in all of the examined milk samples were well below the action level of 500 ng/L permitted by the US regulations. In comparison, several studies have been reported on the contamination of milk and dairy products with AFM_1 (16) reported that the incidence of AFM_1 in UHT milk in Portugal was 84.2%, ranging 5 to 61 ng / L. A survey from Turkey, (17) reported that AFM_1 was detected in 67% of the UHT milk samples. During 1996, 161 samples of milk in Italy checked for Aflatoxin M_1. AFM_1 was detected in 125 (78%) of milk samples (ranging from ‹1 to 23.5 ng/l; mean level 6.25ug/l) (18). In Kuwait, 54 samples of dairy products were analyzed for Aflatoxin M_1. Of samples, 28% were contaminated with AFM_1 with 6% being above the maximum permissible limit of 0.2 ug/l (19).

Table 2: - Levels of Aflatoxin M1 in local and imported sterilized milk:-

Source of UHT milk	Sample analyzed	Positive samples	AFM1 concentration (ng/L) range	Means	Exceed legal limit (%)
Local milk					
1	**4**	**3(75%)**	**N.D-20.15**	**11.9**	**0.0**
2	**4**	**3(75%)**	**N.D- 100**	**36.93**	**25.0**
3	**4**	**3(75%)**	**5-160**	**48.03**	**25.0**
Imported milk					
1	**18**	**17(94%)**	**N.D – 84**	**37.55**	**28.0**
2	**6**	**6(100%)**	**6.55 – 60.60**	**28.00**	**33.0**
3	**6**	**4(67%)**	**N.D – 25.93**	**11.03**	**0.0**
Total	**42**	**36(86%)**	**N.D - 160**	**29.00**	**17.0**

Libyan and EC limits is 50 ng/L

N.D= not detected

These results are in parallel with the findings of some previous reports (20, 21, 22, 23, 24) which pointed out the presence of AFM_1 in more than 60% of the UHT milk samples in Turkey.

The wide variations in AFM_1 levels among studies could be related to geographic and climatic differences but also to differences in feeding systems, and farm management practices (25).

It was clearly observed that the milk which is processed into dairy products (local and imported sterilized milk) may contain a high concentration of AFM_1 and/or be contaminated with *Aspergillus* spp. Consequently, this subject is a serious problem for the public health since all the age groups including children consume these products worldwide. For this reason, milk and dairy products have to be inspected continuously for AFM_1 contamination.

CONCLUSIONS

Results of this study indicated that the levels of AFM1 in raw milk produced in Elbaida city were very low (less than 10 ng/l) compared to sterilized milk (17%) exceeding the Libyan regulation (50 ng/l). The levels of AFM1 in raw milk and local nd imported UHT milk should be controlled and important to maintain low levels of AFB1 in feeds of dairy animals.

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