Assessment of Aflatoxin M1 Residues in Raw Cow Milk at Al- Riyadh Area with Reference to Some Detoxification Applications

Yosef, T.A.[1*]; Al- Julaifi, M.Z.[2]; Salah-El-Dein W.M.[3] and AL-Rizqi, A.M.[2]

[1]Dept. of Forensic Med. and Toxicology, Fac. of Vet. Med., Kafrelshiekh Univ., 33516, Egypt.
[2]Toxicology lab. Management of Vet. Laboratories, Min. of Agric, Riyadh, 11418, KSA.
[3]Animal Health Research Inst., Dept. of Food Hygiene, Zagazig Provincial Lab., 44516, Egypt.
tarektoxicology@yahoo.com

Abstract: This study was carried out to evaluate the levels of aflatoxin M1 (AFM1) in sixty raw cow milk samples collected from different farms at Al- Riyadh area, Saudi Arabia, besides reviewing the reduction effects of some detoxification methods on it. Results of the field study revealed that the mean concentration of AFM1 was 0.185 ±0.0181 ppb. On the other hand, 43 (71.7%), out of 60 examined samples, contained AFM1 residues in levels exceeded the EU maximum limit for raw milk (0.05 µg/l). Meanwhile 32 (53.3%), out of 60 samples, surpassed the Gulf maximum limit for raw milk (0.2 µg/l). For experimental study, negative milk samples for AFM1 were mixed and divided into 4 main groups which inoculated with 10, 5, 2.5 and 1.25 µg/l AFM1 standard respectively. Each group subdivided into 4 subgroups of 5 samples (100 ml each). The 1st subgroup let as control, the 2nd subgroup undergo pasteurization at 65°C for 30 minutes following by sudden cooling at 4°C, the 3rd subgroup treated by boiling at about 100°C for 10 minutes; while, the 4th one exposed to microwave radiation for 2 minutes in microwave oven at high energy level. The obtained results exhibited a significant reduction in AFM1 concentrations by all treatment methods comparing with the actual positive control levels. The reduction rate were ranked as follow: microwave radiation exposure (52.08%) > boiling treatment (23.93%) > pasteurization treatment (12.90%). Accordingly, microwave irradiation of AFM1 contaminated cow milk may be valuable to reduce its levels and subsequently minimize its hazardous on the public health.
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1. Introduction

Cow milk is defined as the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, containing not less than 8.25% milk solids-not-fat, and not less than 3.25% of milk fat **(Eskin, 1990)**. Milk is a part of common diet for adults as well as children which are more susceptible to the hazardous effects of toxins as AFM1 which furthermore, can be passed from raw milk to milk products **(Diaz *et al.*, 1995)**.

Aflatoxins are toxic mold metabolites produced by toxigenic strains of *Aspergillus* species. They have an important role in the occurrence of some human diseases such as liver cancer, chronic hepatitis and cirrhosis **(IARC, 2002)**. Aflatoxin B1 (AFB1) is the most commonly occurring and the most acutely toxic among aflatoxins **(Etzel, 2002)**. Alongside, AFB1 is the most potent hepatocarcinogen known in mammals.

Aflatoxin B1 is degraded in the rumen of dairy cows and minor but important part is resorbed and metabolized in liver into AFM1 (4-hydroxylated metabolite of AFB1) which now considered the most threatening aspect of AFB1 contamination. About 0.3–6.2% of AFB1 in animal feed is transformed to AFM1 in milk **(Bozoglu, 2009)**. Aflatoxin M1 is relatively stable and after circulation in blood it is excreted in milk, urine or bile. Excretion of AFM1 in milk may vary from animal to animal, from day to day and from milking to the next. The occurrence of AFM1 in milk is transitory in nature and can be detected in the milk after 12-24 hrs and reaches maximum within two days after the intake of the contaminated commodity **(Ebrahim, 2010)**.

The presence of AFM1 in milk and dairy products can be a potential risk to the health of consumers **(Manetta, *et al.*, 2009).** According to the International Agency for Research on Cancer, the AFM1 are classified as human carcinogens class one **(IARC, 2002 and Food Safety Watch, 2012).** Several countries have established regulatory limits for AFM1 in raw milk and milk products, which vary from country to country **(Ruangwises & Ruangwises, 2010).** The European Community has set the maximum permitted level for AFM1 in raw milk and heat-treated milk at 0.05 µg/l **(EC, 2006).**

Control of aflatoxins is the need of the hour, since their occurrence in foods and feeds is continuously posing intimidation to both health and economics all over the world. Numerous strategies for

their detoxification from food and feed materials have been proposed including physical, chemical and biological agents **(Basappa & Shantha, 1996)**. Boiling and exposing to microwave radiation exhibited an important role to reduce aflatoxin levels in foods **(Farag *et al.*, 1996))**. Also, pasteurization of milk decreased the levels of AFM1 as reported previously by **Hossein *et al.*(2007).**

The aim of the current investigation was to evaluate the levels of AFM1 in raw cow milk samples collected from Al-Riyadh area; more to the point, studying the detoxification outcomes of boiling, pasteurization and microwave radiation treatments on AFM1 in cow milk samples.

2. Material and Methods

I: Field study

A: Sampling

Sixty raw cow milk samples (1 liter each) were collected from different farms at Al- Riyadh area, Saudi Arabia during spring and summer 2012s. Throughout transportation, the milk samples were kept in ice packets in an icebox. Samples were identified, stored in deep freezing at -18ºC and protected from light until analysis has been conducted in Toxicology Lab, Ministry of Agriculture, Saudi Arabia.

B: Quantitative determination of AFM1

The amount of AFM1 was determined according to Enzyme-Linked Immuno Sorbent Assay, (ELISA), method by using the Ridascreen® aflatoxin M1 (R-Biopharm AG, Darmstadt, Germany) test kit which is a competitive enzyme immunoassay based on antigen-antibody reaction **(Karimi *et al.*, 2007).** The milk samples were centrifuged for 10 minutes at 3500 rpm in 10°C. The upper creamy layer was completely removed by aspirating through a Pasteur pipette. Exactly 100 µl of skimmed milk was used directly in each well for quantitative test. A sufficient number of micro titer wells were inserted into the micro well holder for standards and prepared samples. The steps of ELISA examination were conducted automatically by using ChemWell® fully automated ELISA instrument. Exactly 100 µl of the standard solutions and prepared samples were added in separate wells and they were incubated for 60 min at room temperature in the dark. The liquid was poured off the wells and the micro well holder was tapped upside down vigorously (three times). All the wells were filled with 250 µl of washing buffer and emptied as described earlier. The washing procedure was repeated twice. After that, 100 µl of the enzyme conjugate was added and incubation for 60 min at room temperature in the dark was done. The washing sequence was repeated three times. Substrate and chromogen solutions were added to each well and mixed thoroughly (50 µl each), followed by incubation for 30 min at room temperature in the dark. After the incubation time, 100 µl of stop reagent was added to each well and mixed completely. Finally, the measurement of AFM1 was done photometrically at wavelength of 450 nm. All the previously mentioned steps and the calculation of the final results were conducted automatically by the ChemWell® Automatic ELISA software.

II: Experimental study

A- Milk samples inoculation

Aflatoxin M1 negative milk samples (total volume about 10 liter) were mixed, divided into 4 main groups, and inoculated with 10, 5, 2.5 and 1.25 µg/l AFM1 standard respectively.

B- Aflatoxin M1 standards

Aflatoxin M1 standards from SIGMA-ALDRICH® 10 µg/ml acetonitrile were used in the present experiment in concentrations described above.

C- Treatment of the inoculated samples

Each group subdivided into 4 subgroups of 5 milk samples (100 ml each). The 1st subgroup let as control. The 2nd subgroup subjected to pasteurization at 65°C for 30 minutes followed by sudden cooling at 4°C **(Pasteurization process, 2012).** The 3rd subgroup treated by boiling at about 100°C for 10 minutes **(National Dairy Council, 1993)**. The 4th subgroup exposed to microwave radiation in microwave oven at high energy level for 2 minutes **(Zhao *et al.*, 2012).**

D- Assessment of AFM1 concentrations

The concentrations of AFM1 were estimated in all subgroups (80 samples) as previously described in the field study.

III: Statistical analysis

Statistical analysis of data was conducted using "Statistic for animal and veterinary science" **(Petric and Watson, 1999).**

3. Results and Discussion

Table 1, exhibited the levels of AFM1 in raw cow milk samples collected from Al- Riyadh area, Saudi Arabia. Concerning our results, the mean concentration of AMF1 in raw milk was 0.185±0.0181 ppb. This result was higher than those previously recorded in raw milk by **Lopez *et al.*(2003)**, **Sugiyama *et al.* (2008), Kang & Lang (2009) and Al Zuheir & Abu Omar (2012)** in Argentina, Japan, Kenya and Palestine, who estimated AFM1 concentrations of 0.016, 0.011, 0.12 and 0.029 ppb respectively. Furthermore, in pasteurized milk, lower AFM1 level (0.05 ppb) than our assessments was detected in Iran by **Fallah (2010)**.

Table 2 revealed that, the ratio of AFM1 positive samples was 71.7% (43 out of 60 samples). This ratio coincided with that estimated in Brazil by **Shundo & Sabino (2006)** and in Iran by **Fallah (2010)**, who detected 74.4% and 72.5% as AFM1 incidences in the examined samples respectively.

Meanwhile, higher rate than our figures were detected in Argentina by **Lopez *et al.* (2003)** and in Palestine by **Al Zuheir & Abu Omar (2012)** who recorded 89% and 85% as AFM1 positive samples respectively. Contrarily, in marketed milk, in Portugal, **Duarte *et al.* (2013)** appraised lower incidence of AFM1 positive samples (27.5%) than that recorded in the current study.

Regarding the relation between our estimations and the permissible limits, the gained results (Table 2) showed that, all the positive samples (71.7%) exceeded the AFM1 EU maximum limit for raw milk (0.05 ppb). Previous studies by **Fallah (2010)**, **Al Zuheir & Abu Omar (2012) and Duarte *et al.* (2013)** recorded 36.2%, 20% and 5% as incidences of milk samples go above the EU maximum limit respectively. On the other hand, our records showed 32 (53.3%) out of 60 samples exceeded the AFM1 Gulf maximum limit for raw milk (0.2 ppb).

Results of our survey declared that, all the positive samples affirmed relatively high levels exceeded the EU maximum limits, in spite of its incidence was moderate when compared with the previous studies as mentioned above. This fluctuation may be explained by the appropriate suitable environmental conditions for spoilage fungi in Saudi Arabia as the climatic and storage conditions of the tropical and subtropical countries are most favorable for the development and growth of aflatoxigenic fungi in food and feed stuffs. Over and above, the aflatoxigenic *Aspergilli* are generally regarded as storage fungi, proliferating under conditions of relatively high moisture/humidity and temperature. Aflatoxin is produced at a temperature of 12-40 °C and requires 3-18% moisture **(Duncan & Hagler, 2008).** These conditions can be come to pass during transportation, processing and storage of imported animal feed ingredients, the main source of animal feed in Saudi Arabia beside local dried green fodders. The geographical distribution and climatic variations can influence AFM1 occurrence and contamination levels in milk **(Galvano *et al.*, 1996 and D'Mello & Macdonald, 1998).**

Results in Tables 3 and 4 clearly showed a significant reduction in the levels of AFM1 in all samples by the three treatment applications applied in this study. In all different examined milk groups, the more significant reductions in the levels of AFM1 were recorded in samples exposed to microwave radiation (52.1%) followed by samples treated by boiling (23.9%) which go after that subjected to pasteurization (12.9%), except in group III there is no significant variations in AFM1 levels between actual positive control and pasteurization treatments; also, in group IV, the difference between AFM1 concentrations in boiling and pasteurization groups were insignificant.

Previous studies concurred with our findings, as reported by **Samarajeewa *et al.* (1990),** who indicated obvious elimination of aflatoxins, arrived to 100%, in peanut meal by γ radiation. Also, **Iqbal *et al.*(2012)** reported 6% reduction of aflatoxin in hot peppers after exposure to γ irradiation. Moreover, **Farag *et al.* (1996)** recorded significant reduction of the pure total aflatoxin and aflatoxin in grains after exposure to microwave irradiations. In addition to **Ajoy & Priyanka (2010)** who stated that, microwave roasting destroyed aflatoxins almost completely in the harvested crops. On the other hand, **Samarajeewa *et al.* (1990)** recorded 50% reduction of total aflatoxin in crude peanut oil after heating at 120°C for 10 min. Moreover, **Choudhary *et al.* (1998)** reported that sterilization of milk at 121°C for 15 min caused 12.21% degradation of AFM1, whereas boiling decreased AFM1 by 14.50%. Also, **Soliman et al, (2001)** indicated that boiling reduced aflatoxin percentage in rabbit liver by a range of 67% to 80%. Additionally, pasteurization plays a role in the aflatoxin reduction as previously reported by **Bakirci (2001)**, who reported that pasteurization caused a decrease in the level of AFM1 at the rate of 7.62%. Also, **Deveci (2007)** showed that pasteurization can partially reduce the amount of AFM1 in milk. Furthermore, **Hossein *et al.* (2007)** recorded AFM1 level of 8.7 ppb in pasteurized milk samples which was 24.2 ppb in raw milk samples collected from the same area.

From aforementioned results, we could be concluded that the AFM1 level in milk was reduced by microwave radiation, boiling and pasteurization as described previously. The most obvious detoxification effect obtained by microwave radiation exposure. Thus, exposure of cow milk to the microwave radiation may be precious to reduce the levels of AFM1 and subsequently diminish its perilous on the public health.

Table 1. The mean concentrations (ppb) of aflatoxin M1 in raw cow milk samples collected from different farms at Al- Riyadh area, Saudi Arabia, (n=60).

Minimum	Maximum	Mean	±S.E.
ND*	0.455	0.185	0.0181

*ND: not detected

Table 2. The frequency distribution of aflatoxin M1 residues in raw cow milk samples collected from different farms at Al- Riyadh area, Saudi Arabia, (n=60).

Milk samples	Positive samples		ND		European limit (0.05 ppb)*				Gulf countries limit (0.2 ppb)**			
					Below PL		Over PL		Below PL		Over PL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
	43	71.7	17	28.3	17	28.3	43	71.7	28	46.7	32	53.3

NB: ND: not detected; PL: permissible limit.
* EC, (2006).
** Standardization Organization for G.C.C. (1997).

Table 3. The mean concentrations (ppb) of aflatoxin M1 in different treated milk samples

Initial AFM1 levels	Detected mean levels of AFM1 in the different subgroups			
	Actual positive control levels	Pasteurization treatment	Boiling treatment	Microwave radiation treatment
Group I (10 ppb)	9.0 ±0.311[a]	7.8 ±0.177[b]	6.7 ±0.354[c]	4.2 ±0.399[d]
Group II (5 ppb)	4.5 ±0.12[a]	3.8 ±0.169[b]	3.2 ±0.132[c]	2.3 ±0.177[d]
Group III (2.5 ppb)	2.4 ±0.04[a]	2.3 ±0.051[a]	1.9 ±0.164[b]	1.1 ±0.086[c]
Group IV (1.25 ppb)	1.08 ±0.048[a]	0.88 ±0.037[b]	0.86 ±0.081[b]	0.52 ±0.071[c]

Values in the same row with different superscript are significantly different at $p < 0.05$.

Table 4. The mean detoxification % of aflatoxin M1 in different treated milk samples

Initial AFM1 levels	Actual positive control levels	The mean detoxification % of AFM1 after treatments		
		Pasteurization treatment	Boiling treatment	Microwave radiation treatment
Group I (10 ppb)	9.0 ±0.311	13.3	25.6	53.3
Group II (5 ppb)	4.5 ±0.12	15.6	28.9	48.9
Group III (2.5 ppb)	2.4 ±0.04	4.2	20.8	54.2
Group IV (1.25 ppb)	1.08 ±0.048	18.5	20.4	51.9
Mean %		12.90	23.93	52.08

Corresponding Author
Tarek A. A. Yosef,
Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Kafrelshiekh University, Kafrelshiekh 33516, Egypt. tarektoxicology@yahoo.com.

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