The Occurrence of Aflatoxin M1 in Milk, soft cheese and yoghurt in Baghdad Province by Using ELISA Test

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Accepted on: 26/10/2013

Summary

Milk and dairy products are fundamental components in the human diet and may be the principle way for the entrance of Aflatoxin M1 (AFM1) into the human body. All milk and dairy products samples were tested for the occurrence of AFM1 by the competitive ELISA technique. Out of 32 bovine raw milk samples that were collected from eight villages around Baghdad province, 32 samples (100 %) were contaminated with AFM1 ranging from 0.15 to 86.96ng/kg with mean value of 42.37±26.07 ng/kg, of which 17 samples were contaminated with concentrations < 50 ng/kg and 15 samples exceeded the maximum acceptable level of AFM1 in milk (50 ng/kg) imposed by the European legislation. The raw milk samples belonged to animals fed with composite and stored fodder as in Althahab Alabiadh, Radhwaniya and Fadhaliya villages had higher significantly AFM1 concentrations over all the other five villages (Grazing feed). All 32 (100%) locally produced soft white cheese samples analyzed were contaminated with AFM1 ranging from 31.84 to 89.44 ng/kg with the mean value of 59.92±17.03 ng/kg. Out of 32 locally produced yoghurt samples analyzed, 32 samples (100%) were contaminated with AFM1 ranging from 0.16 to 42.74 ng/kg with the mean value of 16.92±11.55 ng/kg. Thirty samples (100%) of the examined 30 imported UHT milk samples collected from different commercial companies in the province of Baghdad presented significantly high contamination level with AFM1 that were found to range from 0.18 to 85.66 ng/kg.

Keywords: Aflatoxin M1, Milk, Soft cheese, Yoghurt, ELISA.

Introduction

Aflatoxin M1 (AFM1) is the hydroxylated metabolite of Aflatoxin B_1(AFB_1) that can be found in milk and milk products obtained from livestock that have ingested contaminated feed with AFB1(1). The AFB1, once ingested by the animal, is rapidly absorbed by the gastrointestinal tract and is transformed into the metabolite AFM1, which appears in the blood after 15 minutes and is then secreted in the milk by the mammary gland (2). There is sufficient evidence that AFM1 is carcinogen, genotoxic and is less toxic thanAFB1 (3 and 4). The potential hazardous of human exposure to AFM1 via consumption of milk and milk products has been demonstrated (5). To protect consumers from contaminated dairy products, considering that milk and milk derivatives are consumed daily and, moreover, that they are of primary importance in the diet of children, most countries have set up maximum admissible levels of AFB1 in feed (6) and of AFM1 in milk, which vary from the 50 ng/kg established by the EU, to the 500 ng/kg established by USFDA (7 and 8). The maximum admissible level in fresh milk and dairy products is not established yet in Iraq, and few data about AFM1 incidence on fluid milk and other dairy products have been published. Monitoring surveys were frequently conducted in several countries all over the world to determine the level of AFM1 in milk and milk products.

There are different analytical methods used for Aflatoxin determination including thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), immuno-sensors, immunochemicals methods, such as enzyme linked immunosorbent assay (ELISA), which is the most widely used test to detect aflatoxins due to its simplicity, sensitivity and adaptability (9). The purpose of this study was to determine the status of AFM1 contamination in milk and milk products around and within Baghdad city.

Materials and Methods

The raw milk and imported milk (UHT) and dairy products samples (cheese and yoghurt) were collected randomly at weekly intervals in sterile 100 ml plastic bags from various regions of Baghdad province such as (Althahab, alabiath, Fethaliya, Rathwaniya, Ghazalia, Sabiat, Altaji and Alameria). The samples were conserved cooled in an ice box during the transfer to the laboratory. Milk and Dairy products samples were stored in a refrigerator (4^0C) until the analysis was conducted in the laboratory.

Five ml of fresh raw milk sample (full-cream milk) were pipetted into test tube and incubated for 30 minutes at (4^0C). The sample was centrifuged at 3000g for 10 minutes. 450ul of the clear milk serum below the fat layer were removed and mixed with 50ul methanol. This solution was directly measured in the ELISA assay (10).

A representative cheese sample was crushed in a mixer without the addition of liquid. 2g of the cheese sample were combined with 10ml of mixture of Hexane, Methanol, and double distilled water (50:30:20) and agitated for 30 minutes on a horizontal shaker at 125rpm. The liquid was decanted and centrifuged for 5 minutes at 3000g.The lower aqueous methanolic phase was removed by means of a Pasteur pipette. This liquid was diluted 1:10 with sample buffer. This solution was directly measured in the ELISA assay (11).

Yoghurt samples were diluted in buffer (phosphate buffer saline –PBS PH: 7.2) by 1:5 ratio. After mixing, 100 microliter of the solution was used for ELISA. (12 and 13). Aflatoxin M1 contain 10ug/1ml acetonitrile toxin (Sigma, Germany), the acetonitrile was evaporated by incubator at 37^0C for 24 hours. Then 10 ml of methanol was added and kept in the dark conditions at 2-4^0C (14).

All samples were prepared as described above. 100ul of diluted standards, blank and prepared samples were pipetted in duplicate into the appropriate wells of the microtiter plate and immediately 50 ul Aflatoxin M1 antibody was added into each well. The microtiter plate was covered with a plastic foil and incubated for 60 minutes at room temprture on a microtiter plate shaker (or 90 minutes without shaker).The plate was washed three times as follow: the contents of the well was discarded (dump or aspirate) and 300 ul of diluted wash Buffer were pipetted into each well and after the third repetition the wells were emptied again and removed residual liquid by striking the plate against a paper towel. The wash procedure is critical because insufficient washing will result in poor precision and falsely absorbencies. 100ul of IgG (HRP) were pipetted into each well. The microtiter plate was covered with a plastic foil and incubated for 60 minutes at room temperature on a microtiter plate shaker (or 90 minutes without shaker). The plate was washed as outlined in above. 100ul of Substrate (TMB) were pipetted into each well. The reaction was allowed to develop in the dark (e.g. cupboard or drawer; the chromogen is light –sensitive) for 20 minutes at room temperature. Enzyme reaction was stopped by adding 100ul of stop solution into each well. The blue color was turned yellow upon addition. After thorough mixing, the absobance was measured at 450nm, using an ELISA reader, the color was stable for 30 minutes.

Results and Discussion

The amount of the Aflatoxin M1 contents in 32 raw milk samples that were collected randomly from individual producers homes (50 L milk cans) of Althahab Alabiadh, Radhwaniya, Fadhaliya, Al-Taji,Ebrahim bin Ali , Sabbiat, Ghazaliya, and Ameriya are shown in Table 1. The raw milk samples that were collected from Althahab Alabiadh, Radhwaniya, Fadhaliya and Ghazaliya had significantly ($P<0.01$) higher AFM1 concen-tration over all other villages with the mean values of 68.12±14.26, 63.19±13.06, 59.82±16.76 and 52.54±8.36 ng/kg respectively. This latter contamination levels could be hazardous because they exceeded the legal maximum acceptable level of 50 ng/kg according to European Legislation.

The contamination level with AFM1 in the above four mentioned villages could be attributed to the bad management conditions especially that related to dairy feed quality since the quality of fodder used for feeding the

dairy cattle is important and the ingestion of food contaminated with Aflatoxin B1 by the dairy cattle was the way of exposure to Aflatoxin M1 in milk (15).

The raw milk samples that were collected from both Al-Taji and Ebrahim bin Ali had significantly (P<0.01) the lowest levels of AFM1 concentration over all the above four mentioned villages with the mean values of 18.14±20.70 ng/kg and 0.32±1.72 ng/kg respectively. This latter low levels of contamination with AFM1 could be attributed to the influence of animal feeding (grazing feed) on the assumption that animals fed on pasture would be less exposed to AFB1 ingestion and consequently, would produce less AFM1 contaminated milk. The contamination level of AFM1 in milk samples that were collected from both Sabbiat and Ameriya were found to be low with the mean values of 36.62±14.66 and 38.02±8.66 ng/kg respectively which did not exceed the legal maximum acceptable level of 50 ng/kg according to European Legislation.

Table, 1: Occurrence of Aflatoxin M1 in raw milk samples (50 L milk can) and its concentration (ng/kg).

Source of samples	No.of samples	No.of positive samples	Range ng/kg	Mean±SD ng/kg	
Althahab Alabiadh	**4**	**4**	**55.26 -86.96**	**68.12 ± 14.26**	**A***
Radhwaniya	**4**	**4**	**44.7 - 73.68**	**63.19±13.06**	**A**
Fadhaliya	**4**	**4**	**47.72 - 83.96**	**59.82±16.76**	**A**
Al-Taji	**4**	**4**	**0.15 – 37.6**	**18.14±20.70**	**C**
Ebrahim bin Ali	**4**	**4**	**0.24 – 0.40**	**0.32±1.72**	**D**
Sabbiat	**4**	**4**	**18.22 – 52.80**	**36.62±14.66**	**B**
Ghazaliya	**4**	**4**	**41.75 -61.11**	**52.54±8.36**	**A**
Ameriya	**4**	**4**	**31.89 – 44.15**	**38.02±8.66**	**B**
Total	**32**	**32 (100%)**	**0.15-86.96**	**42.37±26.07**	

*** Means not followed by the same letter differ significantly.** **SD Standard Deviation.**

Among the 32 (100%) raw milk samples from the above eight mentioned villages (Table, 1) that were positive for AFM1 only 15 raw milk samples were found to have AFM1 concentrations above the legal maximum acceptable limit in the range of 50.69 to 86.96 ng/kg whereas only 17 raw milk samples were found to have AFM1 concentrations under the legal maximum acceptable limit in the range of 0.15 to 49.18 ng/kg.

The occurrence and levels of AFM1 in 32 locally produced soft white cheese samples that were collected randomly from the individual producer of Althahab Alabiadh, Radhwaniya, Fadhaliya, Al-Taji, Ebrahim bin Ali, Sabbiat, Ghazaliya and Ameriya are shown in (Table, 2).

Statistical evaluation revealed that there was no significant (P>0.05) difference between the mean concentration of AFM1 of cheese samples produced in Althahab Alabiadh and Ameriya and no differences between the mean concentration of AFM1 of cheese samples produced in Al-Taji and Ebrahim bin Ali villages, and between the mean concentration of AFM1 of cheese samples that produced in Radhwaniya, Fadhaliya, Sabbiat and Ghazaliya. The locally produced soft white cheese samples that were collected from Althahab Alabiadh and Ameriya had significantly (P<0.01) the highest AFM1 concentration over all other villages with the mean values of 82.56±4.98 and 83.80±7.97 ng/kg, respectively.

Soft white cheese samples that were collected from Radhwaniya, Fadhaliya, Sabbiat and Ghazaliya had significantly (P<0.01) lower AFM1 concentrations with the mean values of 65.66±13.84, 64.13±6.48, 58.63±12.36 and 56.25±6.82 ng/kg respectively. The AFM1 contamination levels of the above mentioned six villages could be hazardous because they exceeded the legal maximum acceptable level of 50 ng/kg accepted by the European countries. Soft white cheese samples that were collected from both Al-Taji and Ebrahim bin Ali had significantly (P<0.01) the lowest AFM1 concentration over all the above mentioned villages with the mean

value of 40.38±9.88 and 39.95±6.74ng/kg respectively. This latter contamination level did not reach or exceed the legal maximum acceptable level of 50 ng/kg by the European countries. The soft white cheese made from raw milk belonged to grazing dairy cattle as in Ebrahim bin Ali and Al-Taji villages would be less contaminated than the other soft white cheese samples made from raw milk of animals fed with composite and stored fodder as in Althahab Alabiadh, Radhwaniya and Fadhaliya villages.

Table, 2: Occurrence of Aflatoxin M1 in locally produced soft white cheese and its concentration (ng/kg).

Source of samples	No.of samples	No.of positive samples	Range ng/kg	Mean±SD ng/kg	
Althahab Alabiadh	**4**	**4**	**75.82 – 87.51**	**82.56±4.98**	**A***
Radhwaniya	**4**	**4**	**48.68 – 82.78**	**65.66±13.84**	**C**
Fadhaliya	**4**	**4**	**58.26 – 73.38**	**64.13±6.48**	**C**
Al-Taji	**4**	**4**	**32.12 -54.66**	**40.38±9.88**	**B**
Ebrahim bin Ali	**4**	**4**	**31.84 -48.24**	**39.95±6.74**	**B**
Sabbiat	**4**	**4**	**44.24 -71.32**	**58.63±12.36**	**C**
Ghazaliya	**4**	**4**	**49.92 – 65.72**	**56.25±6.82**	**C**
Ameriya	**4**	**4**	**78.16 – 89.44**	**83.80±7.97**	**A**
Total	**32**	**32 (100%)**	**31.84-89.44**	**59.92±17.03**	

*** Means not followed by the same letter differ significantly.** **SD Standard Deviation.**

The variations in the level of cheese contamination by AFM1 may be explained by variation in the original milk contamination due to influence of animals feeding (grazing or composite feed) on the assumption that animals fed on pasture would be less exposed to AFB1 ingestion and, consequently, would produce less AFM1 contaminated milk. Accordingly, cheeses made from milk belonging to grazing animals, would be less contaminated than cheese made from milk belonging to animals fed with composite and stored fodder (16 and 17).

The occurrence and levels of AFM1 in 32 locally produced yoghurt samples that were collected randomly from the individual producer of Althahab Alabiadh, Radhwaniya, Fadhaliya, Al- Taji , Ebrahim bin Ali, Sabbiat, Ghazaliya and Ameriya are shown in (Table, 3). Locally produced yoghurt samples that were collected from Althahab Alabiadh, Radhwaniya, Fadhaliya, Ghazaliya and Ameriya had significantly ($P<0.01$) higher AFM1 concentration over the other villages with the mean values of 22.56±8.98, 25.95±12.86, 24.58±6.08, 24.86±7.06 and 25.52±0.24 ng/kg, respectively.The locally produced yoghurt samples that were collected from Ebrahim bin Ali , Al-Taji and Sabbiat villages had significantly ($P<0.01$) lower AFM1 concentration over all the above mentioned villages with the mean values of 6.11±7.42, 6.86±10.38 and 15.63±4.08 ng/kg, respectively. Locally produced yoghurt made from raw milk belonging to grazing dairy cattles as in Ebrahim bin Ali and Al- Taji would be less contaminated than yoghurt made from raw milk belonging to animals fed with composite and stored fodder as in the Althahab Alabiadh, Radhwaniya and Fadhaliya.

The mean values of AFM1 concentration in yoghurt samples that were collected from all villages were significantly ($P<0.01$) lower than that found in the raw milk samples of the same villages (Table, 1). Cultured dairy products are usually manufactured by adding a starter culture to initiate the fermentation process and probably some strains of Lactic acid bacteria existing in the yoghurt were effective in reducing the AFM1 contamination level from milk (18).

Table, 3: Occurrence of aflatoxin M1 in locally produced yoghurt and its concentration (ng/kg).

Source of samples	No.of samples	No.of positive samples	Range ng/kg	Mean±SD ng/kg
Althahab Alabiadh	4	4	11.92 – 32.72	22.56±8.68 A*
Radhwaniya	4	4	12.41 – 42.71	25.95±12.86 A
Fadhaliya	4	4	19.32 -30.92	24.58±6.08 A
Al-Taji	4	4	0.16 – 22.14	6.86±10.38 C
Ebrahim bin Ali	4	4	0.16 – 15.86	6.11±7.42 C
Sabbiat	4	4	11.66 – 20.93	15.63±4.08 B
Ghazaliya	4	4	18.45 – 33.72	24.86±7.06 A
Ameriya	4	4	19.35 – 31.69	25.52±0.24 A
Total	32	32 (100%)	0.16-42.74	16.92±11.55

*** Means not followed by the same letter differ significantly. SD Standard Deviation.**

The prevalence rate of AFM1 contamination in locally produced yoghurt samples was 100%. In other words, out of 32 locally produced yoghurt samples analyzed, 32 samples (100%) were contaminated with AFM1 ranging from 0.16 ng/kg to 42.74 ng/kg where as the mean value of AFM1 contamination level found in all the positive yoghurt samples was 16.92 ±11.55 ng/kg. This latter contamination level did not exceed the legal maximum acceptable level of 50 ng/kg accepted by European countries.

The occurrence and levels of AFM1 in all 30 UHT milk samples that were collected randomly from different commercial companies in province of Baghdad such as Awj, Hana, Hammoda, Jawda, Nada and KDD are shown in (Table, 4). The UHT milk samples that were collected from Hana, KDD, Hammoda and Jawda companies presented high contamination levels with AFM1 which were found to range from 47.88 -78.59 ng/k, 47.83 -85.66 ng/kg, 39.15 – 84.97 ng/kg and 35.46 – 84.98 ng/kg respectively. While the UHT milk samples that were collected from Nada and Awj companies presented lower contamination levels with AFM1 which were found to range from 0.18 – 62.79 ng/kg and 0.28 – 33.49 ng/kg respectively (Table 4). The UHT milk samples that were collected from Hana, KDD, Jawda and Hammoda companies had significantly ($P<0.01$) higher AFM1 concentration with the mean values of 68.33±15.56, 68.18±12.14, 64.41±20.62 and 64.15±24.30 ng/kg respectively over the other companies.

This latter contamination levels could be hazardous because they exceeded the legal maximum acceptable level of 50 ng/kg that accepted by European countries. The UHT milk samples that were collected from both Nada and Awj companies had significantly ($P<0.01$) lower AFM1 concentration with the mean values of 24.48±21.68 and 14.67±18.91 ng/kg respectively over all other companies. This latter contamination levels did not exceed the legal maximum acceptable level of 50 ng/kg that accepted by the European countries (Table, 4).

Table, 4: Occurrence of Aflatoxin M1 in UHT imported milk samples and its concentration (ng/kg).

Source of samples	No.of samples	No.of positive samples	Range ng/kg	Mean±SD ng/kg
Awj -Syria	5	5	0.28 – 33.49	14.67±18.91 D*
Hana- Syria	5	5	47.88 – 78.59	68.33±15.56 A
Hammoda-Jordan	5	5	39.15 – 84.97	64.15±24.30 B
Jawda-Saudia	5	5	35.46 – 84.98	64.41±20.61 B
Nada-Saudia	5	5	0.18 – 62.79	24.48±21.68 C
KDD- kuwait	5	5	47.83 – 85.66	68.18±12.14 A
Total	30	30 (100%)	0.18-85.66	51.03±28.37

*** Means not followed by the same letter differ significantly. SD Standard Deviation.**

The mean value of AFM1 contamination level found in all the 32 (100%) positive raw milk samples that were collected randomly from eight villages around and within Baghdad province was 42.37 ± 26.07 ng/kg. The locally produced soft white cheese samples that were made from raw milk that belonged to the same eight villages had significantly (P<0.01) higher AFM1 concentration over the raw milk samples and the mean value of AFM1 contamination level found in 32 (100%) positive cheese samples was 59.92 ± 17.03 ng/kg. Several authors have demonstrated that AFM1 was bound to the milk casein (1, 19 and 20). AFM1 is a water –soluble component and due to the hydrophobic side of the casein molecule, AFM1 has the affinity to bind casein of the milk (21). Other researches (22) reported that the AFM1 was more concentrated in the cheese curd than in the milk used to produce it. Several recent papers reported that the highest concentration of the AFM1 was found in the cheese curd than in the milk used to produce it (19, 23-26). The locally produced yoghurt samples that were made from raw milk of the same eight villages had significantly (P<0.01) the lowest level of AFM1 concentration over both raw milk and soft white cheese samples and the mean value of AFM1 contamination level found in the 30 (100%) positive yoghurt samples was 16.92±11.55 ng/kg. This latter low level of contamination with AFM1 could be attributed to the influence of starter culture that initiated the fermentation process and probably some strain of Lactic acid bacteria existing in the yoghurt that were effective in reducing the AFM1 contamination level from milk. These results were in agreement with the results of different authors (27-29). Lactic acid bacteria present in the fermented milk was responsible for the detoxification of AFM1 by biological degradation (30-32). The imported UHT milk samples that were collected from different commercial companies in the province of Baghdad presented significantly (P<0.01) high contamination level with AFM1 with the mean value of AFM1 concentration found in the 30 (100%) positive UHT milk samples was 51.03± 28.37 ng/kg. AFM1 was found to be relatively resistant to the heat treatments and it was almost entirely retained in the pasteurized milk (33).

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انتشار الافلاتوكسين M1 في الحليب والجبن الطري واللبن في محافظة بغداد بإستخدام فحص الأليزا

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الخلاصة

يعتبر الحليب ومنتجاته من المواد التي تدخل في غذاء الانسان كما انها تعتبر الطريق الرئيسي لدخول الافلا M1 في جسم الانسان.تم فحص كافة عينات الحليب ومنتجاته للكشف عن وجود الافلاتوكسين M1 بواسطة اختبار (ELISA) وتبين ان من مجموع 32 عينة من الحليب البقري الخام والتي جمعت من ثمانية مناطق في بغداد , بأن 32 عينة (100%) كانت ملوثة بسموم الافلاتوكسين M1 وبتركيز تراوح مابين0.15 الى 86.96 kg\ng وبمعدل 26.07±42.37kg\ng واظهرت النتائج ان 17 عينة من مجموع العينات 32 عينة كانت ملوثة بسموم الأفلا M1 وبتركيز اقل من الحد القانوني (50> ng\kg) في حين 15 عينة كانت ملوثة بسموم الأفلا M1 وبتركيز اعلى من الحد القانوني (50ng\kg) المسموح به في القوانين الاوربية .ان عينات الحليب البقري الخام التي تم جمعها من ابقار تم تغذيتها على اعلاف مخزونة كما هو الحال في عينات الحليب التي تم جمعها من قرية الذهب الابيض والرضوانية والفضيلية احتوت على التركيز الاعلى لسموم الأفلا M1 وبمستوى معنوي ($P < 0.01$) وبمعدل 14.97±68.12 و 13.06±63.19 و 16.76 ±59.82 kg\ng على التوالي عن باقي القرى التي يتم تتبع نظام تغذية الابقار في المراعي . كما اظهرت النتائج ان جميع عينات الجبن الطري المنتج محليا 32 (100%) والتي تم فحصها كانت ملوثة بسموم الافلا M1 وبتركيز تراوح مابين 31.84 الى 89.44 kg\ng وبمعدل 17.03 ± 59.92 kg\ng. كما وجد من بين 32عينة لبن خاثر منتج محليا ان 32 عينة (100%) كانت ملوثة بسموم الافلا M1 وبتركيز تراوح بين0.16 الى42.74 kg\ng وبمعدل 11.55±16.92kg\ng . أظهرت عينات الحليب المعقم المستورد (UHT) ان 30 عينة (100%) من مجموع 30 عينة والتي تم جمعها من مناشئ تجارية مختلفة من المحلات التجارية في بغداد كانت ملوثة بسموم الافلاتوكسين M1 وبمستوى عالي ومعنوي ($P < 0.01$) وبتركيز تراوح مابين 0.18الى 85.66 kg/ng وبمعدل 28.37±51.03 kg\ng.

الكلمات المفتاحية: الافلاتوكسين M1، الحليب، الجبن الطري، اللبن، فحص الأليزا.