



Aflatoxin M1 detection by ELISA in raw and processed milk in Bangladesh

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ARTICLE INFO

Keywords:

Aflatoxin
Aflatoxin M1
Aflatoxin B1
ELISA
Milk

ABSTRACT

An analysis was accomplished to get information regarding presence of highly toxic and carcinogenic aflatoxin M1 (AFM1) in raw and processed samples of milk applying ELISA (enzyme linked immunosorbent assay). Investigation of a set of 100 samples (n=100) taken from different regional small-scale farms as well as grocery stores of Bangladesh containing three groups of milk including raw (n=50), pasteurized (n=25) and UHT (n=25), exhibited in total 53% AFM1 contamination where 70% contamination was found in raw milk ranging from 22.79–1489.28 ng/kg (mean value 699.07 ng/kg), 52% in pasteurized milk ranging from 18.11–672.18 ng/kg (mean value 99.77 ng/kg) and 20% in UHT milk ranging from 25.07–48.95 ng/kg (mean value 35.46 ng/kg). Among all the positive samples, 75% contaminated samples were above the European Communities prescribed limits (50 ng/kg) while having 25% samples still below this limit whereas 43% samples crossed the accepted limit of US regulations/Codex Alimentarius Commission regulations (500 ng/kg). Thus the findings of this study may lead to increase awareness regarding health impact of aflatoxin M1 and implementation of strict regulations by law enforcement bureaus of Bangladesh.

1. Introduction

Some specific *Aspergillus* species, such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius* naturally produce major toxic mycotoxins named aflatoxins (AFs) which are responsible for causing contamination in plant derivative products [1]. Mainly four aflatoxin classes- (AFB1- aflatoxin B1), (AFB2- aflatoxin B2), (AFG1- aflatoxin G1) and (AFG2- aflatoxin G2) are established based on prolonged drought, humidity and temperatures, composition of substrates, storage time and other crucial factors playing significant role in fungal synthesis of AFs [2,3]. Among them, the International Agency for Research on Cancer has kept AFB1 under “group I” [4,5] due to its high toxicity, teratogenicity, hepatocarcinogenicity and mutagenicity [6]. Aflatoxin B1 is converted into its fundamental hydroxylated metabolite called aflatoxin M1 at the liver of livestock by a superfamily of enzymes named cytochrome P450 and ingestion of feed contaminated with aflatoxin B1 can cause excretion of aflatoxin M1 in milk [7,8]. Depending on toxicity and carcinogenicity, in 2012, IARC has also classified aflatoxin M1 as “group I” [9].

According to in vitro metabolic activation, the potency of aflatoxin M1 to cause cancer was only 10% than AFB1 regarding its carcinogenicity [10] whereas both AFM1 and AFB1 showed similar severe carcinogenicity in ducklings as well as rats both in quantitative and qualitative manners [11]. However, as children consume milk the most

[12–14], presence of even little amount of aflatoxin M1 in raw and processed milk increases the possibilities to be affected by this detrimental toxin [15,16].

Detection of aflatoxin can be done by different vigorous methods including thin-layer chromatography (TLC) [17], liquid chromatography/electrospray-tandem mass spectrometry [18], high-performance liquid chromatography (HPLC) [19]. But due to high cost and troublesome sample preparation procedure, these analytical methods have been replaced with fast and easily detectable assay technique named ELISA for doing regular analysis of both milk and milk based products for the past 20 years [20]. It is noteworthy that ELISA gives specific and quick response with feasible large scale repetition capabilities [21], besides gets first preference based on low cost, rapid action and requirement of small scale volume of sample [22].

From the viewpoint of undesirable and major health concern, there is precise directive for fixing aflatoxin M1 level in products associated with milk in several states all over the world [20]. These regulatory limits may differ between countries as financial affairs do affect them [23,24]. Based on ALARA (as low as reasonable achievable) principle, the highest degree of aflatoxin M1 in liquid formed milk along with dried milk products is 50 ng/kg specified by the European Commission (EC) [25]. Nonetheless, 500 ng/kg is the declared limit of US regulations and Codex Alimentarius Commission (CAC) for fixing aflatoxin M1 level to be

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<https://doi.org/10.1016/j.toxrep.2020.09.012>

Received 7 February 2020; Received in revised form 23 September 2020; Accepted 25 September 2020

Available online 4 October 2020

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present in milk [23,26,27]. In between, the regulatory level in Syria is limited at 200 ng/kg [28]. On the other hand, specifically the scientific panel under the European Commission has announced 25 ng AFM1/kg as the utmost allowable level to be present in formulae, applicable for young children [29]. Besides, different countries apply strict regulations to keep levels of aflatoxins low in livestock feed. For example, 20,000 ng/kg is recommended by the US- Food and Drug Administration (FDA), as the topmost gross aflatoxin concentration acceptable for food together with feed considering the consumption purpose of dairy [30]. The allowable limit for human consumption is much lower than the animal feed, with 4000 ng/kg intended for aflatoxins in total and in case of AFB1, it is 2000 ng/kg [28]. Besides, The EU and EFTA countries maintain 5000 ng/kg for feed utilized by dairy livestock [31].

Being an enriched source of major nutrients such as protein, calcium, iodine and potassium, milk and milk based products have achieved an increased production and consumption rate both in urban and rural areas of Bangladesh like other South Asian countries [32]. But Bangladesh has no separate specific scale for limiting AFM1 and thus the objective of this study is to indicate the necessity to specify a regulatory limit for AFM1 by exploring the degree of its contamination rate both in raw milk as well as processed milk samples in Bangladesh.

2. Material and methods

2.1. Sample description

A complete set of 100 samples (n=100) consisting of raw cow milk (n=50), pasteurized milk (n=25) and ultra-high temperature (UHT) treated milk (n=25) was collected from January to April, 2019 during dry winter and early summer for analysis. Among them, raw cow milk samples were accumulated randomly from different local sites including farms, and processed milk, such as pasteurized and UHT milk samples, of various popular and unpopular trademarks were taken from local area and different grocery shops of Bangladesh on random basis. After collection, either samples were taken for analysis instantly or stocked at -20°C until the time for investigation but no longer than 3–4 days.

2.2. Sample preparations

A commercial ELISA kit (Romer Labs, Singapore) was used to detect AFM1 contamination in the present experiment. At first 5 ml of milk samples were pipetted in test tubes and incubation was performed at 4°C while the time was 30 min. After 10 min centrifugation at 3000 g, the creamy portion settled at the upper side of the tube was separated fully whereas 0.4 ml of milk serum below the fat layer was taken to mix with 0.1 ml of 100% methanol (the ratio between milk serum and methanol was 4:1).

2.3. Aflatoxin M1 analysis of raw and processed milk using competitive enzyme linked immunosorbent assay

For this investigation, AgraQuant aflatoxin M1 sensitive (25/500 ng/kg) kit (Romer Labs) was used. The storage temperature for the kit was $2-8^{\circ}\text{C}$ and was kept at 37°C for one hour before using. According to the test kit instructions: 200 μl of conjugate was taken into each dilution well (supplied with the kit) in a micro well strip holder. 100 μl of each standard (0, 25, 50, 100, 200 and 500 ng/kg) and also samples were added with conjugate through careful pipetting on duplicate manner. Later, from every dilution well, 100 μl solution was transferred into corresponding micro wells coated with antibody and then incubation was done at 37°C for 60 min in unlighted condition. Afterwards, complete removal of liquid was done by pouring out and cleaning the wells through wash buffer for 5 times in the washing steps. Subsequently, substrate solution (100 μl) was taken into the wells where the reaction proceeded in darkness at 37°C for 20 min and blue color was found. In the last step, stop solution (100 μl) was added in to the wells, as a result

the bluish color turned into yellowish one. Finally, the optical density was taken at the recommended wavelength (450 nm) in a specific Microplate reader (MULTISCAN FC) using the software ScanIt for Multiscan FC 3.1. A sheet named log-logit AFM1 (provided with the ELISA kit) was used to create a standard curve and the final concentration of aflatoxin M1 was calculated by putting the absorbance of the samples against the standard curve. According to the kit's instruction, the dilution factor for the calculation of final AFM1 was 1 and the detection limit (LOD) applied for raw along with processed milk samples was 18 ng/kg (if samples containing aflatoxin M1 concentrations ≥ 18 ng/kg were marked as "positive samples" and samples containing aflatoxin M1 concentrations < 18 ng/kg were marked as "negative samples") with recovery rate of 93–119% where coefficient of variation (CV) was 8.5% by three different analysts using two different lots of test kits.

3. Results and discussion

3.1. Analytical evaluation

An experiment in order to observe aflatoxin M1 level in raw and processed samples of milk was done by ELISA technique for having rapid output through user-friendly extraction method with high specificity [33]. Fig. 1 shows the calibration curve found from the present study using the immunosorbent assay. Six concentrations (0, 25, 50, 100, 200 and 500 ng/kg) levels were taken to draw the calibration curve from which the unknown concentrations of the samples could be interpolated.

An analytical evaluation of ELISA was implemented to certify the quality of data through ascertainment of variation coefficient along with recovery. Here, four different concentrations (25, 50, 200, 500 ng/kg) were spiked with fresh milk. The percentages of recovery in spiked samples of milk was found to be as 117% (coefficient of variation is 14) for 25 ng/kg, 102% (coefficient of variation is 9) for 50 ng/kg, 99% (coefficient of variation is 5) for 200 ng/kg and 92% (coefficient of variation is 6) for 500 ng/kg (Table 1).

3.2. AFM1 existence

In total, 100 samples of milk containing 50 raw milk, 25 pasteurized milk and 25 ultra-high temperature (UHT) treated milk were investigated and the occurrence of AFM1 contamination levels are shown in Table 2. Among 100 milk samples, 53% samples were found to be contaminated containing AFM1. The percentages of AFM1 were- 70% in raw milk having the range of 22.79–1489.28 (ng/kg) (mean value 699.07 ng/kg), 52% in pasteurized milk having the range of 18.11–672.18 ng/kg (mean value 99.77 ng/kg) and 20% in UHT milk ranging from 25.07–48.95 ng/kg (mean value 35.46 ng/kg). The possible reason behind the higher aflatoxin M1 contamination rate in raw cow milk compared to other samples is that Bangladesh possesses weather dependent agriculture where summer and winter are the

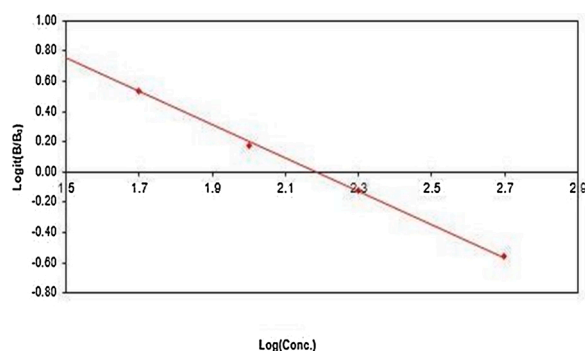


Fig. 1. Standard curve considered for AFM1 where B/B_0 renders 450 nm absorbance.

Table 1

Analytical performance of the method determined by ELISA.

Spiking Levels (ng/kg)	Repetitions	AFM1 (ng/kg)	Recovery* (%)	SD (%)	CV (%)
25	5	29.3	117	4	14
50	5	50.8	102	4	9
200	5	197.2	99	11	5
500	5	458.1	92	28	6

AFM1, aflatoxin M1; CV, coefficient of variation; SD, standard deviation; *Formula: (encountered aflatoxin M1/spiking concentration of aflatoxin M1) × 100.

Table 2

AFM1 levels in raw and processed milk samples.

Types	Sample No.	Positive samples	Presence of AFM1 (%)	AFM1 concentration (ng/kg)	
				Range (ng/kg)	Mean ± SD (ng/kg)
Raw Milk	50	35	70	22.79–1489.28	699.07 ± 469.57
Pasteurized Milk	25	13	52	18.11–672.18	99.77 ± 175.49
UHT Milk	25	05	20	25.07–48.95	35.46 ± 11.02
Total	100	53	53	18.11–1489.28	498.53 ± 488.76

preferable seasons for crop production. In 2016, Ali found that here in Bangladesh, until new crops of a new season arrive, people hugely consume the harvest of the previous season in the next one [34]. As a result, moulds can easily grow on feedstuffs producing mycotoxins due to long storage condition along with high humidity during the harvest time [35] which are taken by lactating cattle further promoting deliberation of aflatoxins in raw milk. The present study shows similarity with another investigation where AFM1 appeared in 28 of total 38 raw milk (73.6%), 17 of total 25 pasteurized milk (68.0%) and 5 of total 14 powdery formed milk (35.7%) [36]. Similarly, a close concurrence is found between the findings of this study and other reports confirming presence of AFM1 in different locally marketed milk samples at comparable or equal values done by previous investigators [37,38]. In addition, aflatoxin M1 contamination was reported in 36 milk samples that ranged from 3 to 64 ng/kg where the mean value was 14 ng/kg [39]. Another monitoring survey on aflatoxin M1 occurrence in raw cow milk acknowledging drought condition of two following years disclosed that AFM1 contamination was more prevalent in drought year rather than a non-drought year [40].

The southern region of Asia, specially Iran, India and Pakistan conducted various studies to reveal aflatoxin M1 contamination level in milk and milk based products [41]. In 2011, Fallah et al. disclosed 323 ng/kg as the mean value of aflatoxin M1 in Iran [42] whereas in 2018, Asghar et al. from Pakistan reported 346.2 ng/kg as mean aflatoxin M1 level in 91.7% fresh milk samples [43]. In 2016, Aslam et al. reported presence of aflatoxin M1 in each and every 468 fresh milk samples where 2600 ng/kg was the mean level [44]. Though occurrence of AFM1 contamination is remarkable in South Asian countries rather than European ones as they possess stringent laws and good storage practices of feedstuffs to control aflatoxins [41], the present results show lower contamination of AFM1 than some other European countries like Greece and Portugal where up to 90% of aflatoxin M1 presence was noticed in raw milk [45,46] respectively. In parallel, 91 milk samples out of total 96 samples collected from the Greek market were found to be contaminated with AFM1 having the mean value of 10 ng/kg [47].

In general, aflatoxins are resistant to heat [8]. However, in 1998, Choudhary et al. experimented the impact of multiple heat treating

methods on aflatoxin M1 in fresh milk and proclaimed 12.21% deterioration due to sterilization that continued for 15 min having the temperature of 121°C, while 14.50% degradation occurred due to boiling [48]. Concluding remark was marked by them as- temperature and time are mainly responsible for causing elimination of aflatoxin M1. Therefore, 20% AFM1 contamination had been found for UHT milk in the present study whereas [49] reported 29.8% AFM1 prevalence rate of UHT milk.

500 ng/kg is the defined limit for aflatoxin M1 considering dairy farm products stated by the US regulations and Codex Alimentarius Commission (CAC) [50,51]. In contrast, 50 ng/kg has been fixed as aflatoxin M1 paramount limit specified by European Communities [52]. Samples that exceeded EC and US/Codex act are shown in Table 3. Here, around 75% of the samples containing aflatoxin M1 ranged from 50.05–1489.28 ng/kg and crossed the limits set by the European Commission, while the US/Codex Alimentarius authorized regulations were surpassed by 43% milk samples that ranged from 739.04–1489.28 ng/kg. In 2004, an experiment done by Rastogi et al. came out with almost 99% (75/76) aflatoxin M1 containing milk products exceeding the European Commission suggested limits, while in the same study, it was found that 9% (7/76) positive samples transcended the US limits [12]. Recently in 2019, Costamagna et al. published an investigation where 5.5% out of 77.8% positive milk samples had higher rate of aflatoxin M1 contamination crossing over the European regulations [39]. In contrast, only 2 traditional milk samples among 91 positive samples overpassed the established limit of European Commission [47]. Noteworthy point is that although ten times greater value for aflatoxin M1 is advised by US regulations, still 43% of milk samples of Bangladesh exceeded the maximum limits indicating the urgency of establishment of day to day analytical surveillance of milk.

In 2017, Gonçalves et al. stated that AFM1 excretion in raw milk is the major consequence of the revelation of cattle to aflatoxin B1 dietary levels [53]. Around 1–2% of AFB1 can be transferred to aflatoxin M1 by ingestion of contaminated feed, though these values may vary up to 6% of the AFB1 intake [54]. In 2015, Xiong et al. proclaimed that multiple factors work behind the aflatoxin M1 excretion amount into milk- AFB1 ingestion levels, milk yield, stage of lactation, mammary gland condition and individual responsiveness [55]. In 2017, Gonçalves et al. also pointed out the useful application of fermentation by-products containing yeast cells, specially cell wall and autolyzed yeast, in minimizing the availability of feedstocks containing aflatoxin B1 and thus the excretion rate of aflatoxin M1 in raw content [53]. In 2018, Omeiza et al. divulged 86.8% AFB1 contamination in total of 144 feed samples in the range of 500–24800 ng/kg using HPLC [56]. Bangladesh has previous report on the incidence of AFB1 contamination found in eight poultry feed and usually consumed food items among which five food commodities exceeded EC regulations (5 µg/kg) [57]. Therefore frequent evaluation of feedstocks and disposal of contaminated feed are highly appreciable steps to be taken in this regard.

4. Conclusion

In Bangladesh, data regarding aflatoxin contamination in milk are scarce since no regular surveillance of mycotoxins exists. In this regard, this study is a reflection of overall occurrence level of aflatoxin M1 in raw as well as processed milk samples of familiar along with non-familiar brands of this country. Therefore, extensive and periodic analytical surveillance is clearly needed to find out the major sources of aflatoxin M1 contamination and to establish AFM1 regulations as well. HACCP (Hazard Analysis and Critical Control Point) system implementation and maintenance of records of all feeds, feeding practices, contamination levels, proper storage conditions are also highly recommended to achieve SDG-3 (Good health and well-being) by assuring the health safety issues.

Table 3

Contamination levels of aflatoxin M1 in raw and processed milk samples transcending European Commission and US/Codex laws.

Categories	Sample No.	Positive samples	Surpassing EC prescribed concentration (50 ng/kg)		Surpassing US/Codex prescribed concentration (500 ng/kg)	
			No*	Range (ng/kg)	No*	Range (ng/kg)
Raw Milk	50	35	34 (97%)	50.05–1489.28	22 (63%)	739.04–1489.28
Pasteurized Milk	25	13	06 (46%)	51.15–672.18	01 (08%)	672.18
UHT Milk	25	05	–	–	–	–
Total	100	53	40 (75%)	50.05–1489.28	23 (43%)	739.04–1489.28

* Data in parenthesis denote % of positive samples crossing over the accepted limits.

Author declaration

Sahana Parveen planned the study and supervised the research work. Nourin Tarannum performed the whole investigation, data analysis and methodology portions. Meher Nigad Nipa and Suvra Das did the validation and software analysis. Nourin Tarannum wrote down the original draft and all the authors reviewed it.

Statement on conflict of interest

There are no conflicts of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The R & D project of Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR) provided the funding of this study. The authors are grateful to IFST and BCSIR authority as well.

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