

DETERMINATION OF LEVEL OF AFLATOXIN IN RAW MILK AND PASTEURIZED MILK SAMPLES

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ABSTRACT: Aflatoxins are toxic compounds produced mainly by different genera of fungi and transferred to milk through contamination which causes life health hazards. The objective of the current study was to govern its existence in different raw and branded liquid milk samples sold in different areas of District Lahore in Pakistan. For this purpose, 80 samples, 40 raw and 40 branded milk samples were obtained during the months of July and August and analyzed to determine the level of AFM1 in milk by using ELISA technique. Typically, the investigative measures go through the subsequent stages: sampling, extraction, determination and quantification. 50% of the samples were positive for Aflatoxin M1, with a range of 49.73-429.61 ppt. Contamination beyond EU permissible limits for raw milk samples was 67.5% with 52.75-429.60 ppt range and 203.42 ppt mean. Samples collected from Shalimar Tehsil were observed more contaminated than others. On the other hand, branded milk contamination beyond EU limit was 30% with range of 49.73-381.04 ppt and 138.53 ppt mean. Consequently, Greater level of AFM1 in raw milk is a communal health risk, but a checking and scrutiny program for Aflatoxin M1 control in milk industry should be established to avert well-being harms.

Key words: Aflatoxin M1; ELISA; carcinogenic; Lahore; pasteurized milk; raw milk.

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INTRODUCTION

Aflatoxins are one family of mycotoxin (secondary metabolites of genera of fungi) which is natural contaminant in food and feed resulting in human health impact. Aflatoxin is combination of words “A” for genus *Aspergillus*, “fla” for the *flavus* species and toxin, for poison (Bakirdere *et al.*, 2014). These fungi generally contaminate cereals for instance wheat, maize, corn, rice, cotton, pulses, spices, beans and dry fruits (Pittet, 1998) (Severns, 2003), and can cause severe human and animal health issues by triggering innumerable impediments such as hepatotoxicity, mutagenicity teratogenicity, and immunotoxicity (Chang PK *et al.*, 1993), (Kensler TW *et al.*, 2011).

More than 20 AFs are observed, however main four are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (Inan F *et al.*, 2007). Among these AFs, AFB1 and AFB2 are the frequently occurring compounds, which after hydroxylation form AFM1 and AFM2, which lead to contamination of milk and dairy goods. AFB1 and AFM1 are labeled as a most potent carcinogen to humans. Several factors like humidity content, water action, impurity level, toxigenic aptitude of fungi, temperature, storing period and kind of substrate effect the production of aflatoxins (Sheng Y *et al.*, 2014).

Aflatoxin poisoning outbreaks have occurred in different countries around the world, from the first

detection of the disease in England in 1960, when it was known as Turkey “X” disease, to more recent outbreaks in dogs in the United States (Lewis L *et al.*, 2005) and humans in Kenya, India, Thailand, and Tanzania (Kamala A *et al.*, 2018). More than 75 dogs perished in the US between March and June 2011 after consuming aflatoxins-contaminated pet chow (Wouters AT *et al.*, 2013). In Kenya, the most severe outbreak in 2004 resulted in 125 deaths. Aflatoxin contamination of food has also been linked to underweight and growth problems in children (Gong YY *et al.*, 2003).

Animals when feed on aflatoxin contaminated plants and crops then process in their liver AFB1 into hydroxylated metabolite AFM1 that later on expelled in feces and milk. About 1-3% AFB1 that become part of body after ingestion of contaminated food is changed into AFM1 (Guerre E *et al.*, 2000).

Milk has been always a key constituent of food as a source of useful nutrients. More than 50 million population of Pakistan is associated with livestock and Pakistan is one of the top 5 milk producing countries. Pakistan is the fourth largest milk producing countries after India, China and the United States, and yearly milk production is about 45 billion (Asi MR *et al.*, 2012).

AFM1 intake exclusively attack the liver. Initial indications of hepatotoxicity by AFM1 include fever, nausea, jaundice and anorexia followed with stomach pain, hepatitis and vomiting; however, acute poisoning cases are unique and odd. Chronic toxicity of AFM1

involves carcinogenicity and immunotoxicity (Fakhri Y *et al.*, 2019).

Tropical and subtropical regions are more susceptible to aflatoxin contamination. Extreme weathers are favorable for *Aspergillus* spp. Growth (Serraino A *et al.*, 2019) Suitable moisture content and temperature play vital role. During severe conditions plants becomes weak so fungus attack chances increase. Various countries all over the world designed AFM1 concentration limit in all milk products (Ansari F *et al.*, 2019).

AFM1 concentration level determination can be achieved by using different techniques like Thin-layer chromatography (TLC), Liquid Chromatography, High-performance Liquid chromatography (HPLC) and ELISA (Zheng P *et al.*, 2006). Due to rapidness and accuracy ELISA is most frequently and vastly used analytical technique for AFM1 detection in dairy products (Lee NA *et al.*, 2004).

MATERIALS AND METHODS

Sample collection: Total milk samples (n = 80) were collected from District Lahore. For fresh milk sample's collection 5 Tehsils of Lahore includes Shalimar, Model Town, Raiwind, City and Cantt were being covered. Randomly 8 spots selected from each Tehsil, representing the overall quality of raw milk in that specific Tehsil. Raw milk samples were collected in sterilized polythene bags and stored in ice-packed cooler during transportation to laboratory. Pasteurized milk samples (n = 40) of various significant and less significant trademarks were purchased from local and super markets of different areas of Lahore. All the samples were labeled and stored at -4 C° in freezer till further analysis for AFM1.

Sample Preparation: Sample preparation was done according ELISA Kit (Agra Quant ® Aflatoxin M1 sensitive Order #: (COKAQ7100) Roomer Lab, Singapore) suggested procedure. 5ml of milk samples pipetted into the test tubes and then incubated at 4 C° for 30 min. After that milk samples centrifuged at 3000g for 10 min with Centrifuge 5804 R to separate creamy layer from defatted supernatant. After centrifugation layer was separated and milk serum was collected. 0.4ml of this milk serum was mixed with 0.1 ml Methanol (4:1) for further analysis.

Quantitative analysis of AFM1 by ELISA: 100 µL of each prepared sample or standards were added separately in dilution microwells with the help of micropipette and tip was changed for each sample and standard. 200 µL conjugate that actually enzyme conjugate aflatoxin was added in all dilution wells. It had capacity to bound with antibody coated wells. Each sample and standard mixed well with the help of micropipette by pipetting mixture up and down thrice. 100 µL of dilution wells mixture

(sample or standard + conjugate) transferred into antibody coated microwells and incubated in the absence of light for 1 hour at 37 °C to allow reaction for further proceeding. Sample AFM1 and conjugate both compete for antibody binding site. Whole solution from antibody coated microwells dropped off and washed with Diluted wash buffer. 100 µL of substrate added in all microwells with the help of micropipette. Subsequently microwell strips incubated in dark for 20 minutes at 37 °C. Color of microwells content turned blue after adding substrate due to interaction between substrate and conjugate enzyme. So, after addition of substrate well containing 0, 25 or 50 ug/l. 100ul stop solution added in all microwells to stop reaction. In result of stop solution addition mixture color changed from blue to yellow or pinkish. Strips read under reader using 450 nm filter. Optical density recorded for all samples with the help of ELISA reader Stat Fax 4700.

Estimated daily intake (EDI) of AFM1: Daily estimated intake level of AFM1 by milk intake was determined through a method that was put forward by Cano Sancho, Ramos, Peris-Vicente, Marin and Sanchis (2010). The estimation of daily intake of AFM1 level by six age groups (2-4, 5-9, 10-19, 20-39, 40-59, 60 and above) was assessed for 127 persons. This formula was used for EDI calculation of AFM1 (Cano-Sancho G *et al.*, 2010).

$$\text{EDI (ng/kg/day)} = \frac{\text{AFM1 Level in milk (ng/l)} \times \text{Daily milk intake (L/day)}}{\text{Average individual weight (kg)}}$$

RESULTS

Analysis revealed that 50% of the total milk samples were found positive for AFM1. Contamination for raw milk samples was 67.5% with 52.75-429.61 ppt range and 203.42 ppt mean. The AFM1 level in all contaminated raw milk samples was higher than the permissible limit that accepted by the EU. Occurrence of AFM1 contamination in the raw milk samples collected from Cantt, Shalimar, City, Raiwind and Model Town was 62.5%, 87.5%, 75%, 62.5%, % and 50% with range 83.73-327.62, 108.41-429.61, 72.72-338.9, 236.73-414.14, 52.75-186.27 ppt respectively. On the other hand, 32.5% pasteurized milk samples were detected positive with range of 49.73-381.04 ppt and 138.53 ppt mean while contamination beyond EU limit was 30%. Furthermore, data of the milk contamination was further computed to estimate daily intake of AFM1 for six age groups was estimated, which point out that infants are the extremely vulnerable group for AFM1, with 8.91 ng /L per day because high milk consumption and the least affected age group was above 60 years of age with 0.94 mg /L per day.

Analytical Analysis: ELISA technique had been used to determine the AFM1 concentration in raw and

pasteurized milk because of its specificity, sensitivity, quick results and simple procedure. Data was analyzed using Excel 2017 along with Graph Pad Prism 9 and results presented as $MQ \pm SD$.

Incidence of AFM1 in Milk Samples: Total eighty (n=80) samples were collected from Lahore District for estimation of aflatoxins. 40 pasteurized milk samples (P1-P40) were purchased from local and super markets of different areas of Lahore. . While 40 fresh milk samples (R1-R40) were collected from 5 Tehsils of Lahore

includes Shalimar, Model Town, Raiwind, City and Cantt. Randomly 8 spots selected from each Tehsil. The results of the analysis of AFM1 concentration in all milk samples summarized in Table 1. The occurrence of AFM1 was detected in 50% samples. The overall incidence of aflatoxin M1 contamination beyond EU permissible limit of 50ppt was 48.75% having the range of 49.73-429.61 while 1.25 % was within permissible EU limits. Contamination was majorly in raw milk that was 67.5% on the other hand in pasteurized milk was 32.5%.

Table 1: Occurrence of Aflatoxin M1 in Milk samples from District Lahore

Sr. No	Site	Total No. of Samples	No. of Contaminated Samples				No. of Uncontaminated Samples	%	Range
			Within EU Permissible Limits	%	Beyond EU permissible Limit	%			
1	LHR	80	1	1.25%	39	48.75%	40	50.00%	49.73-429.61

AFM1 level in Raw milk Samples: Level of AFM1 in raw milk samples presented in Table 2. The occurrence of AFM1 was detected 67.5% samples. The overall incidence of aflatoxin M1 in all contaminated samples was beyond permissible limit of 50ppt having range 52.75-429.60 ppt with Mean of 203.14 ppt. Across all Tehsils samples collected from Shalimar Tehsil were more contaminated.

Tehsil wise comparison of AFM1 incidence: Occurrence of AFM1 contamination in the raw milk samples collected from Cantt, Shalimar, City, Raiwind and Model Town was 62.5, 87.5%, 75%, 62.5%, % and 50% with range 83.73-327.62, 108.41-429.61, 72.72-338.9, 236.73-414.14, 52.75-186.27 ppt respectively. Results of aflatoxin M1incidence in raw milk samples showed that samples of Shalimar tehsil were more contaminated comparatively than other tehsils.

GraphPad Prism software was used to analyze the data. The one way ANOVA was used to assess data of all tehsils. Differences among variance were considered significant with $P \leq 0.05$.

AFM1 level in Pasteurized milk Samples: Level of AFM1 in pasteurized milk presented in Table 6. The occurrence of AFM1 was detected in 32.5% of all samples. Incidence of aflatoxin M1 in samples was beyond permissible EU limit of 50ppt was 30% and 2.5 % was within permissible limit with range 49.73-381.04 ($MQ(ppt) \pm SD = 138.53 \pm 0.087$)

Comparison of AFM1 level between Raw and Pasteurized Milk: In current study, it was analyzed that raw milk samples were more contaminated by AFM1 while contamination percentage was lower in pasteurized milk samples as compared to raw milk. The occurrence of AFM1 was detected in 32.5% of pasteurized samples. Incidence of AFM1 in contaminated samples was beyond

permissible EU limit of 50ppt was 30% and 2.5 % was within permissible limit with range 49.73-381.04 ($MQ(ppt) \pm SD = 138.53 \pm 0.087$). On the other hand occurrence of AFM1 was detected in 67.5% samples of raw milk that is much higher than pasteurized milk. The overall incidence of aflatoxin M1 in all contaminated samples was beyond permissible limit of 50ppt having range 52.75-429.60 ppt with Mean of 203.14 ppt.

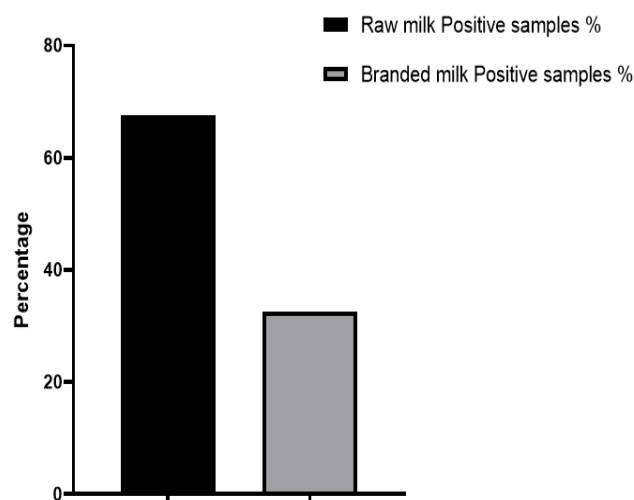


Fig.2: Aflatoxin M1 contamination percentage in Raw and Pasteurized milk samples

EDI of Aflatoxin M1 by different age groups: Daily estimated AFM1 intake by different age groups of the Lahore population is demonstrated. The food frequency questionnaire outcomes shown that age group of 2–4 years are highly exposed to the AFM1 linked health hazards because of their high milk consumption as a solitary diet source. The lowest intake was observed in elders (60 and above age group), where average body

weight is high and intake is less. The results indicate that body weight. Aflatoxin M1 exposure reduce with increase in age and

Table 2: Estimated daily intake of AFM1 incidence by different age groups

Sample Size	Age Group (years)	Average Weight (Kg)	Daily milk intake (L)	AFM1 intake per Day (ng/L/day)
21	2-4	14.37	0.64	8.91
24	5-9	17.82	0.51	5.72
27	10-19	33.63	0.38	2.26
35	20-39	51.94	0.29	1.12
20	40-59	63.04	0.33	1.05
21	60 and above	76.62	0.36	0.94

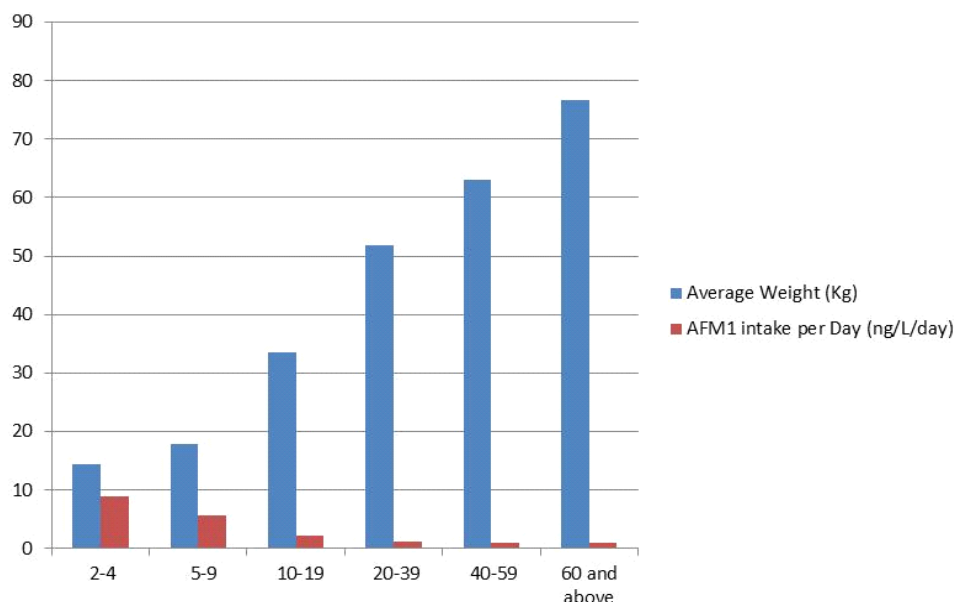


Fig.3: Relationship among estimated daily intake and age

DISCUSSION

Milk is a valuable nutrient containing food that consumed by almost all humans worldwide, the health rate of a country depends upon the food of that country. The contamination of this nutritious food with aflatoxin is an emerging issue which is need to be solve (Prandini A *et al.*, 2009). In developing countries use of substandard contaminated food leads to health related problems in that region (Iqbal SZ *et al.*, 2015). Numerous researches reveal that AFM1 contaminated milk consumption has a promising relation to an increased risk of cancer development in humans (Wang JS, Tang L, 2004). The carcinogenicity of AFM1 may be affected by the period length and concentration level of exposure. Exposure is mainly occurred through the regular consumption of milk and its products (Caloni F *et al.*, 2006).

In Pakistan, a study disclosed that 14% population use pasteurized milk, whereas raw milk is being consumed by 70% population. Additionally,

different kinds of pasteurized milk are prepared from raw milk after various treatments like evaporation, heating etc. Therefore, it's very important to monitor raw milk contamination throughout the year. As Aflatoxin M1 residue level in milk is directly proportion to AFB1 contamination in animal feed. Mostly left over bread also used as feed for cows which act as an important source of aflatoxin in milk (Ismail A *et al.*, 2016). Contamination of milk and its products with aflatoxin is an international issue and international organizations e.g. WHO and FAO are working on this issue and they determined the percentage level of this toxin for contamination. There are two ways by which milk got contamination first mean is AFB1 contaminated feed consumption by lactating animals and second source is contaminated milk when come uncontaminated milk (Salari N *et al.*, 2020). The most common analytical techniques for Aflatoxin quantitative analysis are LC, TLC, HPLC, ELISA, SPE and IAC. ELISA is best quantification technique because of its sensitive and easy procedure (Caloni F *et al.*, 2006).

During this study, raw and pasteurized milk contamination was compared. Results reflect that AFM1 positive samples for raw milk were much higher than pasteurized milk. Among 40 raw milk examined samples 27 were AFM1 positive between the range of 52.75 ppt to 429.60 ppt and in 40 pasteurized milk samples 13 were found positive ranged within 49.73-381.04 ppt. So, 67.5% fresh milk samples detected contaminated with AFM1 on the other hand 32.5% samples of packaged or pasteurized milk were observed positive. Additionally, 100% fresh milk samples and 30% pasteurized samples were above EU permissible limit. Difference of contamination level in fresh and pasteurized milk samples mainly regulatory and monitoring practices that various brands implementing to ensure quality. While, high contamination in raw milk sample primarily due to lack of awareness among local farmers and compromise on feed quality. Fresh milk sample were collected from all Tehsils of District Lahore namely Shalimar, Cantt, City, Model town and Raiwind. 8 random spots selected from each tehsil for sample collection and contamination percentage for these tehsils was 87.5%, 62.5%, 75%, 50% and 62.5%. Contamination percentage was observed high in Shalimar comparatively because sample collection was done in winter season.

Findings of this study were compared with already available statistics of AFM1 prevalence in milk in Pakistan and other countries. In Iran Shush city 120 samples of cow and buffalo milk examined. Samples were analyzed with ELISA technique. Moreover, level of contamination was 69% and 79% with mean 55ng/L, 116ng in raw cow milk and raw buffalo milk respectively (Kamkar A *et al.*, 2014). In Asian Southern region, Abundant research-based studies revealed AFM1 contamination in milk and dairy products specifically in Pakistan, Iran, India, China and Bangladesh. Raw milk contamination found higher than pasteurized or UHT milk (Iqbal SZ *et al.*, 2015).

Fallah reported AFM1 mean value 323 ng/kg in Iran (Fallah AA *et al.*, 2011) while in 2018, Asghar *et al.* reported 91.7% contamination in raw milk samples with mean value of 346.2 ng/kg. (Asghar MA *et al.*, 2018). AFM1 in all 468 fresh milk samples was reported where mean level was 2600 ng/kg (Aslam N *et al.*, 2016). However, contamination of AFM1 in milk is significant in South Asia than European countries as they have rigorous rules and good monitoring system to control aflatoxins M1 (Iqbal SZ *et al.*, 2015). In Portugal and Greece where approximately 90% of aflatoxin M1 occurrence was noticed in fresh milk (Martins ML, Martins HM, 2000). These results revealed high level of AFM1 concentration in Raw milk than current study.

Contamination level in milk was observed 19% (Rodríguez-Blanco M *et al.*, 2020), 11% (Cammilleri G *et al.*, 2019) and 0.8% (Bilandžić N *et al.*, 2016) in Spain, Italy, and Bosnia respectively that is comparatively

significantly less than current study. Various researches show lower contamination of AFM1 in dairy products of European countries.

According to a Serbian study, raw milk in the autumn contained the greatest amount of AFM1, with 29.3 percent raw milk and 4.2 percent milk products above the European Union ML (Miocinovic J *et al.*, 2017). While monitoring the prevalence of aflatoxin content in milk samples throughout the year (Ismail A *et al.*, 2016) investigated the effect of the season, finding that the highest percentage of AFM1 contaminated milk samples (92%) was discovered in the winter season. Various studies have shown that milk produced in the summer is less infected with AFM1 (Peng KY, Chen CY, 2009).

Daily estimated AFM1 intake by different age groups of the Lahore population is demonstrated. The food frequency questionnaire outcomes shown that age group of 2–4 years are highly exposed to the AFM1 linked health hazards because of their high milk consumption as a solitary diet source. The lowest intake was observed in elders (60 and above age group), where average body weight is high and intake is less. The results indicate that Aflatoxin M1 exposure reduce with increase in age, that is parallel with prior studies stated in 2017 (Iqbal SZ *et al.*, 2017). One more research conducted in Pakistan to evaluate AFM1 incidence in milk samples and observed that 87.2% samples were contaminated above the EU and FDA limits, with mean value of 2600 ng /L (Aslam N *et al.*, 2016). Another study reveals that EDI of AFM1 during different months observed almost same, maximum in infants and minimum in adults that is in line with results of current study (Škrbić B *et al.*, 2014).

Conclusion: Milk is an important part of diet for all age groups because of its nutritional value and subsequently milk consumption is rising as human population is increasing day by day. This study reflects incidence of AFM1 in milk in Lahore District, collected at random basis from farms and markets of all tehsils. It was concluded that milk in Lahore is not free of aflatoxins. Results show that raw milk samples are highly contaminated compared to pasteurized samples. Due to high intake of milk by infants they are more vulnerable to AFM1 related health hazards. Highly efficient approach to limit AFM1 in milk is to decrease contamination of AFB1 in lactating animal feed by means of improving agriculture, transportation and storage practices. This research work grants sound foundation for health and food regulatory authorities of Pakistan to initiate distinct measures for continuous regulating and monitoring of AFM1 in milk. Firm permissible limits should be executed to prevent aflatoxin contamination. Awareness campaign and trainings for the farmers and milk retailers may also be useful in this regard.

Higher level of AFM1 in raw milk is a significant health risk, It is essential to enhance feed quality and storage conditions in order to reduce AFB1 production in feed along with AFM1 levels in animal milk. Moreover regular monitoring and scrutiny program for Aflatoxin M1 control in milk industry should be established to avert well-being harms.

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