



Original Article



Identification and Estimation of Aflatoxin M1 in Milk Powder, Pasteurized Milk, and Raw Milk from the Local Market Lahore by ELISA

Muhammad Khalid Saeed^{1*}, Naseem Zahra¹, Hafiza Kainat Anwar², Amara Khan³, Muhammad Zeeshan Kazim³, Ayesha Ijaz⁴ and Ijaz Ahmad¹

¹Food and Biotechnology Research Center, Pakistan Council of Scientific and Industrial Research Laboratories Complex, Lahore, Pakistan

²Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan

³Institute of Food Science and Nutrition, Gomal University, Dera Ismail Khan, Pakistan

⁴Department of Nutrition and Health Promotion, University of Home Economics, Lahore, Pakistan

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*Corresponding Author:

Muhammad Khalid Saeed
Food and Biotechnology Research Center,
Pakistan Council of Scientific and Industrial
Research Laboratories Complex, Lahore, Pakistan
rosefbr1@gmail.com

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ABSTRACT

Because milk has a great nutritional content and can be utilized to make functional dairy products, it is regarded as the ideal natural food for consumers of all ages. It does, however, carry the greatest danger of contaminating human diets with aflatoxin M1. The primary oxidized metabolite of the carcinogenic aflatoxin B1 is aflatoxin M1. It may be caused by feed carry-over contamination and is present in milk and other dairy products. **Objectives:** To measure the amount of aflatoxin M1 in samples of raw and branded milk that were obtained from the Lahore local market. **Methods:** Due to its excellent sensitivity, selectivity, and user-friendliness, the ELISA approach was used to measure aflatoxin M1 in milk for regulatory compliance. **Results:** The results of the investigation showed that AFM1 was found in seven out of ten samples of raw milk, two out of five samples of pasteurized liquid milk, and three out of five samples of brands of milk powder. Aflatoxin levels in positive samples ranged from 0.06 to 0.35 parts per billion. Additionally, validated, the ELISA kits demonstrated high recovery rates and comparable performance. Additionally, it was discovered that the Limit of Detection (LOD=0.01 ppb) levels were substantially lower than the Maximum Residue Limit (0.5ppb). **Conclusion:** From these results, it was concluded that the majority of milk samples were found to be contaminated with aflatoxin M1.

INTRODUCTION

According to the most recent economic report for the fiscal year 2023-2024, Pakistan's livestock industry is the largest contributor to the agricultural value in the country's GDP. The livestock industry alone accounts for 14.36% of the GDP and 60.84% of agricultural output [1]. The scarcity of high-quality feed is one of the most significant obstacles to the growth of our livestock sector. Fodder crops, bushes, grasses, and agro-industrial wastes are the nation's main sources of livestock feed [2]. There are several worries for

livestock breeders. Concerns about feed safety are raised by the fact that mycotoxins or fungal toxins frequently contaminate feed sources in damp and humid environments. Since prehistoric times, milk has been one of the most popular and healthful foods consumed by humans. Because milk has a high nutritional content and can be utilized to make functional dairy products, it is regarded as an ideal natural food for consumers of all ages. There are numerous advantages to milk's nutritional



fortification. Since milk is the primary source of fat, protein, and minerals, it is a complete food. Milk is a valuable nutritional and financial resource because of its solid components, which are primarily lipids and proteins [3]. *Aspergillus flavus* and *Aspergillus parasiticus* species are the main producers of aflatoxins, which are mycotoxins or fungal metabolites [4]. Based on their ultraviolet fluorescence, aflatoxins can be divided into about six major categories. While aflatoxin G1 and aflatoxin G2 produce green fluorescence, aflatoxin B1 and aflatoxin B2 produce blue fluorescence. 4-Aflatoxin M1 and M2 are produced from hydrated AFB1 and AFB2, respectively [5]. The amount of AFB1 ingested and the amount of AFM1 released in milk were shown to be directly correlated. Between 0.3% and 6.2% of the AFB1 that animals consume is transferred into milk as AFM1. Pasteurization, autoclaving, heat inactivation, and other food processing techniques do not affect AFM1 [6]. Hepatotoxicity, malignancy, immunological suppression, nutritional disruption, and teratogenesis in humans are all caused by AFM1. Additionally, growth impairment and immunological suppression might result from exposure to AFs. It has been established that Aflatoxin M1 is somewhat less hazardous than Aflatoxin B1, and the order of their toxicity is AFB1+AFM1 > AFB1 > AFM1 [7]. For these reasons, strict guidelines for AF regulation were taken into consideration in the most recent Commission Regulation of the European Union (EU) no. 2023/915. AFM1 was added to milk at a dosage of 0.05 mg/L [8]. Therefore, maintaining feeds free of AFB1 contamination is crucial to producing high-quality milk free of aflatoxin M1.

There is currently little scientific data regarding the risks associated with the presence of AFM1 in milk that has likely been exposed to feed containing AFB1 in Lahore, Pakistan. This study to determine the prevalence and concentrations of AFM1 in milk powder, pasteurized milk, cow and buffalo milk marketed in the Lahore market, as well as any potential carcinogenic risks associated with its use among Lahore residents.

METHODS

This study employed a cross-sectional laboratory-based design from Nov 2025 to Dec 2025. Five samples of pasteurized milk, five samples of milk powder, five samples of buffalo milk, and five samples of cows' milk were collected from various parts of Lahore (n=10 samples were collected from Allama Iqbal Town, and the remaining n=10 were collected from Nishtar Town). Sample size was calculated using the cross-sectional prevalence formula with a 95% confidence level, 70% expected prevalence (based on a previous Pakistani study [7]), and 5% margin of error, yielding a minimum of 19 samples. To account for non-detectable values, a total of 20 milk samples were collected. Total milk samples (n=20) were collected for

analysis. The milk powder and UHT pasteurized milk samples were bought from various supermarkets in Lahore, while the loose raw milk (cows and buffalo) milk samples were collected from various milk shops in sterile sampling vials and stored in a refrigerator for further analysis. Detection: Aflatoxin M1 was detected in milk samples using competitive ELISA (BIO-RAD; Modal 680, Germany). The Neogen 8632 Veratox® HS aflatoxin M1 test kit's instructions were followed to prepare the samples. The initial step in separating the fat from the milk was centrifugation, which was carried out for ten minutes at 3500 g and 10°C. Following centrifugation, the pipette was used to remove the fat layer, and the lower phase liquid was then used directly in the analysis [9, 10]. AFM1 levels in all milk samples were determined in units of ppb. Quantification in the milk samples was performed using the standard curve ranging from 0.025 to 0.75 ppb. The lower detection limit was 0.01 ppb according to the test protocol. For samples with non-detect (ND) values, a concentration equal to half the detection limit (0.005 ppb) was assigned for statistical analysis [11]. Test procedure: Each well received 50µL of milk samples. Each well received 50µL of the AFM1 standard solution. Each well received 50µL of the enzyme conjugate. After that, 50µL of anti-aflatoxin M1 antibody solution was added. After gently mixing it, it was allowed to sit at room temperature for ten minutes. This was incubated for one hour in the dark. In order to properly clean up the well, the liquid was then poured out over the absorbent paper. The wells were cleaned twice using washing buffer. 250µL of washing buffer was utilized. Each well was then filled with 50 µL of stop solution, gently mixed, and the absorbance was measured at 450 nm [10]. Quality control: Aldrich Chemicals provided a standard solution of AFM1 (A6428) for results validation. Standard AFM1 solution was added to AFM1-free milk samples at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, and 0.75 ppb. The range of AFM1 recovery rates was 92.8-98.9% (Table 1).

Table 1: ELISA Method Efficiency Verification by Spiking Various Levels of AFM1 in Milk

Spiked AFM1 (ppb)	Observed Value (ppb)	Recovery (%)	Coefficient of variance (%)
0.025	0.0232%	92.8%	0.25%
0.05	0.0484%	96.8%	0.50%
0.10	0.0980%	98.1%	0.99%
0.25	0.246%	98.4%	0.64%
0.50	0.494%	98.8%	0.25%
0.75	0.742%	98.9%	0.75%

SPSS version 25.0 was used for data analysis. The variables' frequencies are shown as n (%), and their descriptive statistics are shown as mean ± standard deviation. When comparing groups that had statistically significant differences from one another as a result of the ANOVA, Duncan's multiple comparison test was employed. In every

statistical analysis, results with a p-value less than 0.05 were deemed statistically significant [11].

RESULTS

Aflatoxin M1 levels in branded powder milk, pasteurized liquid milk, and raw milk samples were measured using the ELISA technique, which produced quick results using an easy-to-use extraction process with good specificity. Table 1 displays the mean AFM1 level values for raw milk (n=20) and milk samples from various brands. One-way ANOVA revealed a significant difference in AFM1 concentrations among the milk sample groups ($F(3,16) = 8.42, p = 0.002$). The 95% confidence intervals for mean AFM1 concentrations were 0.11–0.19 ppb for raw milk, 0.05–0.13 ppb for pasteurized milk, and 0.07–0.16 ppb for milk powder. All of the studied samples had AFM1 levels between 0.06 to 0.35 ppb. Additionally, validated, the ELISA kits demonstrated high recovery rates and comparable performance. Furthermore, it was discovered that the Maximum Residue Limit (MRL) (0.5ppb) was much higher than the Limit of Detection (LOD) and Limit of Quantification (LOQ) values (Table 2).

Table 2: Concentration of AFM1(ppb) in Various Milk Samples

Sr. No.	Brand Codes (Specification of Samples)	Aflatoxin-M1 Concentration (ppb)
1	Brand-A (Branded Milk Powder)	0.15±0.009
2	Brand-B (Branded Milk Powder)	ND*
3	Brand-C (Branded Milk Powder)	0.10 ±0.006
4	Brand-D (Branded Milk Powder)	0.09±0.01
5	Brand-E (Branded Milk Powder)	ND*
6	Brand-F (Pasteurized UHT Liquid Milk)	0.14 ±0.009
7	Brand-G (Pasteurized UHT Liquid Milk)	ND*
8	Brand-H (Pasteurized UHT Liquid Milk)	0.08±0.002
9	Brand-I (Pasteurized UHT Liquid Milk)	ND*
10	Brand-J (Pasteurized UHT Liquid Milk)	ND*
11	Brand-K (Cow Raw Milk)	0.12 ±0.008
12	Brand-L (Cow Raw Milk)	0.31±0.022
13	Brand-M (Cow Raw Milk)	ND*
14	Brand-N (Cow Raw Milk)	0.21±0.02
15	Brand-O (Cow Raw Milk)	ND*
16	Brand-P (Buffalo Raw Milk)	0.18±0.01
17	Brand-Q (Buffalo Raw Milk)	ND*
18	Brand-R (Buffalo Raw Milk)	0.35±0.025
19	Brand-S (Buffalo Raw Milk)	0.06 ±0.001
20	Brand-T (Buffalo Raw Milk)	0.27±0.018

n=20, ND* Not detected, and data are represented ± SD. The lower detection limit of the AFM1 test kit was 0.01 ppb. *Significant difference between groups at $p \leq 0.05$

Twenty (n=20) milk samples total ten (10) raw, five pasteurized UHT liquid, and five branded milk powder were examined, and the amounts of AFM1 contamination are displayed in Fig. 1. According to the study's findings, AFM1 was detected in three out of five samples of brands of milk

powder (60%), two out of five samples of pasteurized liquid milk (40%), and seven out of ten samples of raw milk (70%) (Figure 1).

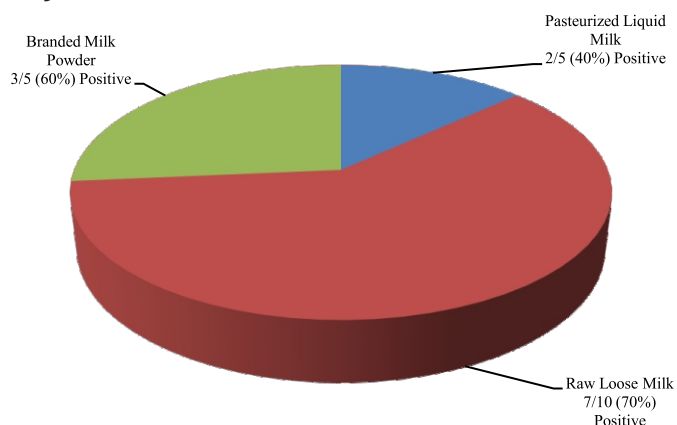


Figure 1: Percentage of Contaminated Milk Samples by ELISA

DISCUSSION

The Competitive Enzyme Linked Immunosorbent Assay (ELISA) method was utilized to identify AFM1 in milk using the Aflatoxin M1 test kit. For the quantitative study of AFM1, the Competitive Enzyme Linked Immunosorbent Assay (ELISA) is an efficient, fast, and dependable method. Aflatoxin M1 (AFM1), a toxin that can have negative health consequences on humans, is released into the milk of animals fed diets tainted with aflatoxin B1. The prevalence of AFM1 in dairy products varies depending on a number of circumstances, such as crop contamination with mycotoxins, inappropriate agricultural methods during harvest, storage, or transport, and excessive humidity prior to and following harvest, which encourages fungal development [12]. Hepatic microsomal cytochrome P450 in the livers of ruminants (cows, goats, sheep, etc.) converts aflatoxin B1 (AFB1)-contaminated feed into aflatoxin M1 (AFM1) [13]. As AFM1 has been known to suppress the immune system, induce changes in DNA structure, and, at high doses, lead to severe health problems in humans such as liver cirrhosis, hepatocellular carcinoma, and stunted growth in babies, it is a severe health hazard to humans and is thus expelled into their milk [14]. Maggira et al. determined the Aflatoxin M1 in Raw Milk Using an HPLC-FL method in comparison with commercial ELISA Kits application in raw milk samples from various regions of Greece and found that raw milk has maximum contamination with aflatoxin M1 [15]. In a different study conducted in Bangladesh, 100 samples (n=100) of different regional small-scale farms and grocery stores in Bangladesh revealed a total of 53% AFM1 contamination, 70% contamination in raw milk with an average of 22.79–1489.28 ng/kg (mean value Among the positive samples, 43 percent of the contaminated samples were above the allowed limit of the US rules/Codex Alimentarius Commission regulations (500 ng/kg), and 75 percent of the

infected samples were above the limits provided by the European Community (50 ng/kg) [16]. The risk assessment demonstrated that the estimated daily intake of 2.7 ng/kg bw/day of the children consuming the AFM1 contaminated milk gave an estimated margin of exposure (MOE) of 0.07 compared to the benchmark dose (BMDL10) of 0.2 ng/kg bw/day. This MOE falls well below the safe threshold of 10,000, indicating a significant carcinogenic and immunotoxic risk despite compliance with EU regulatory limits. These findings underscore the need for routine monitoring and mitigation strategies to protect public health, particularly in vulnerable pediatric populations. Public health implications were assessed by estimating the daily intake of AFM1 from contaminated milk. Using the mean AFM1 concentration in positive samples (0.18 ppb) and assuming a daily milk consumption of 200 mL for adults and 300 mL for children, the estimated daily intakes were 0.036 µg for adults and 0.054 µg for children. These values correspond to 0.72% and 1.08% of the tolerable daily intake (TDI) of 5 ng/kg body weight per day established by the Joint FAO/WHO Expert Committee on Food Additives, based on a 60 kg adult and a 20 kg child. Although all contaminated samples remained below the EU regulatory limit (0.05 ppb), the presence of AFM1 in 60% of samples indicates chronic low-level exposure, which poses a potential cumulative risk, particularly for children who consume more milk per unit body weight and are more susceptible to the immunotoxic and growth-suppressive effects of aflatoxins. Dairy cows and dairy buffaloes may consume more AFB1-contaminated feed during this time, as well as variations in feed storage and diet composition and rainfall effects [17]. The causes of this increased prevalence of AFM1 in raw milk during the winter are not well understood. When contaminated feed is consumed, about 1% to 2% of AFB1 can be converted to aflatoxin M1, albeit this can range up to 6% of the AFB1 intake. According to Gonçalves *et al.* the main effect of cattle being exposed to aflatoxin B1 dietary levels is AFM1 excretion in raw milk [18]. Aflatoxins are generally heat-resistant. However, Choudhary *et al.* tested the effects of various heat-treating techniques on aflatoxin M1 in fresh milk in 1998 and reported that sterilization, which lasted for 15 minutes at 121°C, caused 12.21% deterioration, while boiling caused 14.50%. As a result, the milk powder was contaminated with aflatoxin M1. They concluded that the removal of aflatoxin M1 is mostly caused by temperature and time. According to a study by one scientist [19], aflatoxin M1 contamination exceeded European limits in 5.5% of the 77.8% of milk samples tested. Another researcher [20] also revealed higher levels of AFM1 in cow milk samples from Pakistan, reporting that 53% of samples were above the EU maximum limit for AFM1 in milk (0.5 ppb) and 93% of a total of 520 milk samples tested positive for AFM1.

The number of samples and the inability to compare the AFM1 levels in the milk samples sold in various parts of Pakistan were the study's limitations. Raising public knowledge of the risks associated with feeding milk-producing animals tainted with aflatoxin. It's also critical that storage spaces, temperature, humidity, light, and other factors are appropriate for the feed in question. Long-term research, including a greater number of milk samples, will provide more specific information to the body of knowledge regarding the presence of mycotoxins in milk.

CONCLUSIONS

According to the current study's findings, 60% of milk samples tested positive for aflatoxin M1, yet no sample had a value greater than that of the European Commission. Buffalo Raw Milk (Brand-R) had the highest concentration (0.35 ± 0.025 ppb), whereas Pasteurized UHT Liquid Milk (Brand-H) had the lowest (0.08 ± 0.002 ppb). In order to prevent any serious and irreversible health consequences, the presence of AFM1 in milk samples necessitates monitoring and the application of laws.

Authors' Contribution

Conceptualization: MKS

Methodology: MKS, NZ

Formal analysis: HKA, AK, AI

Writing and Drafting: MZK, IA

Review and Editing: MKS, NZ, HKA, AK, MZK, AI, IA

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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