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Prevalence of Aflatoxin M1 (AFM1) in Fresh Cow Milk Produced in Chattogram, Bangladesh

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A R T I C L E I N F O	A B S T R A C T
	This study was carried out to examine the prevalence of AFM1 contamination across different areas of Chattogram, Bangladesh, and to assess the level of AFM1 in raw milk samples from various dairy farms. A cross-sectional study was conducted to assess the farming standards of three different Chattogram neighborhoods—Bakalia, Khulshi, and Pahartali—and to ascertain the amount of AFM1 in milk. In the study location, 30 commercial dairy farms were randomly chosen, and data on farming methods, milk production techniques, and knowledge of aflatoxin contamination were gathered. Using the enzyme-linked immunosorbent assay (ELISA) method, collected milk samples (n = 90) were examined for the presence of AFM1. The farms produced 71.67±14.71 liters of milk on average every day, which was primarily supplied to dairy processing plants. For feeding the cows, all farms used concentrates and forage. The mean concentration of AFM1 in milk samples collected from Bakalia was higher (190.00±120.87 ng/L) than that in milk samples collected from the Khulshi (108.44±66.19 ng/L) and Pahartali (189.25±160.78 ng/L). The overall prevalence of AFM1 was 43% (N=39) of the total examined samples. A total of 69% (N=27) and 5% (N=2) of AFM1 positive samples exceeded the European Union Regulation (50 ng/L) and BSTI/BFSA regulations (500 ng/L) respectively. There was a significant difference in the occurrence of AFM1 in Bakalia regarding Khulshi and Pahartali. This research will aid in measuring the AFM1 content in raw milk and helping to address public health issues.

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Introduction

Mycotoxins are mold's secondary metabolites that have been linked to a variety of diseases in both animals and humans. Aflatoxins are poisonous compounds generated by fungi such as *Aspergillus*, *Penicillium*, and *Fusarium* spp. (Galvano et al., 2001). AFM1 is a hydroxylated metabolite of AFB1 that may be found in milk and milk products (Bahrami et al., 2016; Xiong et al., 2018). As a metabolite of AFB1, AFM1 is formed in the liver of an animal when AFB1-contaminated feed is consumed (D'Mello and Macdonald, 1997). The AFM1 may be discovered in milk 12 to 24 hours after the initial AFB1 ingestion; its concentration would then rise over the following few days. After 72 hours, the concentration of AFM1 in the milk drops to undetectable levels, indicating

that the AFB1 consumption has been completed (van Egmond, 1989). The dose of AFB1 and the amount of AFM1 excreted in cow's milk had a linear relationship (Battacone et al., 2003). AFB1 in animal feed is converted to AFM1 in milk at a rate of 0.3–6.2% (Creppy, 2002). When farm animals are fed aflatoxin-infected feed, meat and meat products become contaminated with aflatoxins (Huchchannanavar and Balol, 2011). AFM1 causes a variety of devastating disorders in both people and animals. In humans, AFM1 causes hepatotoxicity, cancer, nutritional disruption, immunological suppression, and teratogenic effects (Williams et al., 2004). AFB1 and AFM1 are categorized as class 1 and 2B (or likely) human carcinogens by the International Agency for Research on

Cancer (IARC, 1992). The genotoxic activity of AFM1 was found to be high, however, it was lower than that of AFB1 (Lafont et al., 1989). AFM1 has been discovered in both raw and processed milk products, and it is generally stable and unaffected by pasteurization, Ultra-High-Temperature (UHT) treatment, or processing. The most effective way to reduce aflatoxin exposure is to ensure that foods ingested have the lowest possible aflatoxin concentration, which can be accomplished by putting regulatory limitations on commodities meant for food and feed (Unusan, 2006; Zinedine et al., 2007). Nearly all developed countries have set maximum permitted levels of AFB1 in meals and feeds and maximum permissible levels of AFM1 in milk and milk products to reduce the risk of aflatoxins. AFM1's limits are now widely varied, depending on the country's level of development and economic standing. AFM1 levels in liquid milk and dry or processed milk products should not exceed 50 ng/L, according to several European Community and Codex Alimentarius guidelines (Codex Alimentarius Commissions, 2001). However, according to US regulations, the level of AFM1 in milk should not exceed 500 ng/L (Stoloff et al., 1991). Similarly, Bangladesh Standards and Testing Institution (BSTI) and Bangladesh Food Safety Authority (BFSA) set the AFM1 permissible limit in milk as 500 ng/L. Milk and milk products, which are mostly consumed by youngsters, are high in several nutrients such as proteins and calcium. According to the United Nation's Food and Agriculture Organization (FAO), at least 25% of the world's food crops are contaminated by mycotoxins, and agricultural commodity output is barely keeping up with the world's growing population (Zinedine et al., 2007). Therefore, AFM1 in milk is a cause for concern. As a result, determining AFM1 levels in milk and dairy products is critical to safeguard consumers of various ages from its possible dangers (Fallah, 2010). Considering the foregoing, the current study was conducted to measure the level of AFM1 in fresh raw milk samples from several dairy farms in Chattogram, Bangladesh.

Materials and Methods

Study site and collection of milk samples

This study was conducted throughout the summer and rainy seasons (April – May) in Bangladesh. In the Chattogram Metropolitan Area (CMA), 30 commercial dairy farms from Bakalia, Khulshi, and Pahartali thana were chosen at random. From each area, ten commercial dairy farms were chosen randomly. Three different milk bulk storage tanks were used to gather milk samples from each farm. Following that, 90 milk samples (n = 90) were taken from these three locations. Each milk sample was assigned a unique identification number (about 10 mL). The samples were then promptly transferred to the laboratory using an icebox.

Participatory survey

A prepared questionnaire was used to collect data on owner characteristics (gender, age, education), farm characteristics (number of animals and species), feeding habits, milk production, and respondent's awareness of mold and aflatoxin contamination throughout the sample collection period.

Preparation of milk samples

Romer Labs in Singapore provided us with a commercial ELISA kit (AgraQuant AFM1 fast 100/2000 ng/L and AgraQuant AFM1 sensitive 25/500 ng/L). The AFM1 in the obtained milk samples was detected using this ELISA kit. A total of 5 ml of milk was incubated for 30 minutes at 4°C before being centrifuged for 10 minutes at 3000 Xg. AFM1 was detected directly in the milk serum beneath the fat layer using an ELISA kit.

Enzyme-linked immunosorbent assay (ELISA) of milk samples

AFM1 was measured in milk using a commercial ELISA kit (Romer Labs, Singapore), as directed by the manufacturer. Each well (100μl/well/standard) was pipetted with AFM1 antibody-coated microtiter plate and AFM1 standards (provided with the kit). In addition, duplicate test samples (100μl/well/sample) were pipetted. The samples were incubated for 20 minutes at room temperature on a plate. AFM1 conjugate was added to the wells after a cleaning step with a washing solution (provided with the kit), and the plate was incubated at room temperature for another 30 minutes. To eliminate the unbound conjugate, the plate was washed with the washing solution. A 100 μl substrate solution was added to the wells, and the reaction was allowed to run for 10 minutes at room temperature in the dark, resulting in the development of a blue color. When 100μl of stop solution was added to the wells, the reaction was stopped, and the color changed from blue to yellow. In a Multiskan Ascent ELISA Plate Reader (Thermo Lab Systems, USA), the absorbance was measured at 450 nm, and the absorption intensity was shown to be inversely related to the concentration of AFM1 in the samples. A standard calibration curve was built using different concentrations of AFM1 to calculate the concentration of AFM1 in the sample (Figure 1). Recovery procedures were used with spiked samples in varied concentrations of AFM1 (25, 50, 100, 200, and 500 ng/L) to estimate the accuracy of AFM1 detection. Table 1 shows the analytical efficacy of this ELISA method to detect AFM1. This ELISA's limit of detection (LOD) for fresh milk was 18 ng/L.
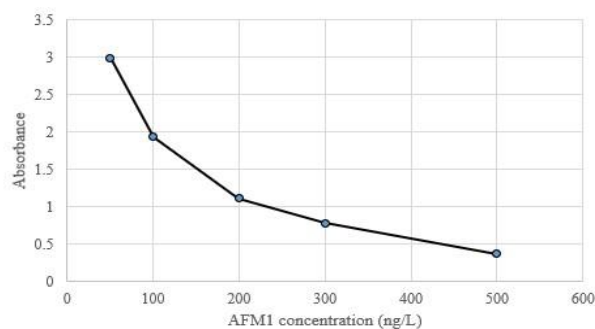

Figure 1. Calibration curve for AFM1 at 450 nm.

Table 1. Analytical efficacy of the ELISA method

Spiked AFM1 (ng/L)	Replications	AFM1 (ng/L)	Recovery (%)	SD	CV (%)

25	3	25.6	102.4	0.7	2.73
50	3	50.2	100.4	1.0	1.99
100	3	94.73	94.73	1.45	1.53
200	3	193.47	96.73	3.36	1.74
500	3	490.33	98.07	2.18	0.44

Table 2. Knowledge and practices related to AFM1 contamination in milk

Variables (N=30)	Frequencies	
1. Knowledge about milk tests	Yes	= 11, No= 19
2. Cold storage system	Yes	= 0, No= 30
3. Knowledge about milk contamination	Yes	= 12, No= 18
4. Occurrence of milk spoilage	Yes	= 24, No= 6
5. Knowledge about AFM1	Yes	= 2, No= 28
6. Knowledge of AFM1 causes in milk	Yes	= 2, No= 28
7. Knowledge about mold growth in feeds	Yes	= 8, No= 22
8. Inspection of feeds before serve	Yes	= 2, No=28
9. Routine animal health check-ups by a veterinarian	Yes	= 3, No= 27
10. Controlled feed storage system	Yes	= 0, No= 30

Statistical analysis

The data gathered in the lab was entered into Microsoft Excel 2010 spreadsheets. The result of AFM1 in different areas of Chattogram was expressed using descriptive analysis. The significance threshold was set at $P<0.05$.

Results

Rate of response and owner's characteristics

A total of 30 farms took part in the survey, resulting in a 100% response rate (n = 30). The owners' ages ranged from 27 to 76, with the majority (77%) being men and the remainder (23%) being women. Among the owners, about 22% of men and 29% of women owners had attained primary-level education (Figure 2).

Farm characteristics

This study featured a total of 30 farms, each of which had a variety of breeds. The most frequent species of these farms were Holstein-Friesian (39.8%) and Jersy (13.2%) cattle. Other types of species were Shahiwal (11.3%), Harians (7.2%), and Pabna cattle (9.5%). In this survey, almost 13% of farms (n = 4) retained adult male cows in the farm. Each farm had an average of 25 (n = 30) animals, with milking cows accounting for 74.5%, dry cows 44.2%, heifers 38.3%, and calves and weaners 68.7%.

Production and distribution of milk

In most farms, milking was done twice a day (morning and evening). The farms included in this study produced 71.67±14.71 liters of milk on average each day. The milk-producing farms in the Khulshi area had higher milk production (77.5±17.04 L/day) than Bakalia (69.2±12.31 L/day) and Pahartali (68.3±14.08 L/day) (Figure 3). Hotel stores, dairy processing plants, and bulking traders were the most common buyers of milk.

Feeding system

Dairy cattle were typically fed dry paddy straw, concentrate (cattle meal, cotton seed cake, and maize germ), molasses, wheat bran, and mustard oil cake. Dairy feeds from commercial feed mills were used by the majority of the farms. All the farms practiced zero-grazing.

The feeding practices of the farms are shown in Figure 4. Most farms lacked a dedicated feed storage facility; thus, grain was stored on the ground next to the farmhouse. Temperature, humidity, mold growth, pest infestation, and other factors in the storage of feed were not monitored by farmers.

Awareness and practices of farmers about aflatoxin contamination

Aflatoxin contamination is a typical occurrence due to poor feed storage facilities. Most of the farmers (93%) were unaware of aflatoxin. The remaining farmers (7%) who had heard of aflatoxin were unsure how to define it. Among the 30 dairy farms, 63% of the farmers do not know about milk safety tests and 100% of the farmers store the collected milk in clear plastic or aluminum bucket under normal environmental conditions (Table 2). Farmers (93%) used to provide dairy feed to cows without inspecting the condition of the feed that had been stored and they have no controlled feed storage system at all (Table 2). The majority of farmers don't have any concerns about animal feed storage systems and mold growth as well as the aflatoxin contamination in feeds (Table 2). This may be a reason for AFM1 contamination in milk so far.

Assessment of AFM1

A total of 90 raw milk samples were analyzed with ELISA. In Table 3, the prevalence and concentrations of AFM1 in raw milk samples from several dairy farms in Bakalia, Khulshi, and Pahartali were statistically analyzed. The overall prevalence of AFM1 was 43% (N=39) of the total examined raw milk samples (Table 3). A total of 11 AFM1-positive milk samples were found among 30 collected samples from Bakalia. The aflatoxin M1 (AFM1) contamination levels were between 24.78–400.96 ng/L with a mean of 190.00±120.87 ng/L. Among the AFM1 positive samples, 82% (N=9) samples exceeded the permissible limit of AFM1 prescribed by the European Union (EU) but all AFM1 positive samples were below the BSTI/BFSA permissible limit (500 ng/L). A total of 17 AFM1 positive samples were examined in Khulshi. In these positive milk samples, the AFM1 contamination levels were between 23.08 - 217.33 ng/L. The mean

concentration of AFM1 found in the samples was 108.44±66.19 ng/L and 59% (N=10) of samples exceeded the permissible limit of AFM1 prescribed by the European Union (EU) and all were below the BSTI/BFSA limit. A total of 11 AFM1-positive samples were found in the Pahartali area where the level of AFM1 contamination was between 29.82 - 533.83 ng/L. The mean concentration of AFM1 found in the positive milk samples was 189.25±160.78 ng/L and 73% (N=8) of samples exceeded the permissible limit of AFM1 prescribed by the European Union (EU) and 18% (n=2) of these positive samples exceeded the BSTI/BFSA permissible limit. The results of the investigation showed no correlation between milk production levels and the prevalence of AFM1 in milk (Figure 4).

Table 3. Statistical Summary of Aflatoxin M1 Level (ng/L).

Study Area	Sample No.	Positive Samples (%)	AFM1 level (Mean ± SD)	Minimum	Maximum	Exceeding EU Limit* (%)	Exceeding BSTI/BFSA Limit** (%)
Bakalia	30	11 (36%)	190.00±120.87[a]	24.78	400.96	9 (82%)	0 (0%)
Khulshi	30	17 (57%)	108.44±66.19[b]	23.08	190.15	10 (59%)	0 (0%)
Pahartali	30	11 (36%)	189.25±160.78[a]	29.82	533.83	8 (73%)	2 (18%)
Total	90	39 (43%)	153.95±119.09	23.08	533.83	27 (69)	2 (5%)

Means followed by different superscripts (a,b) are significantly different (P<0.05). *European Union Permissible Limit (50 ng/L).**Bangladesh Standards and Testing Institution (BSTI) and Bangladesh Food Safety Authority (BFSA) Permissible Limit (500 ng/L).
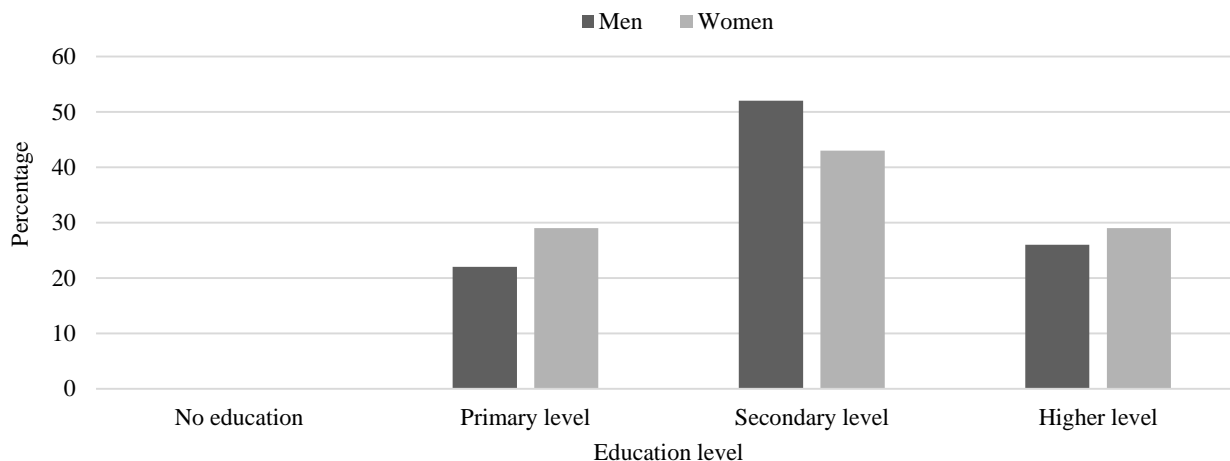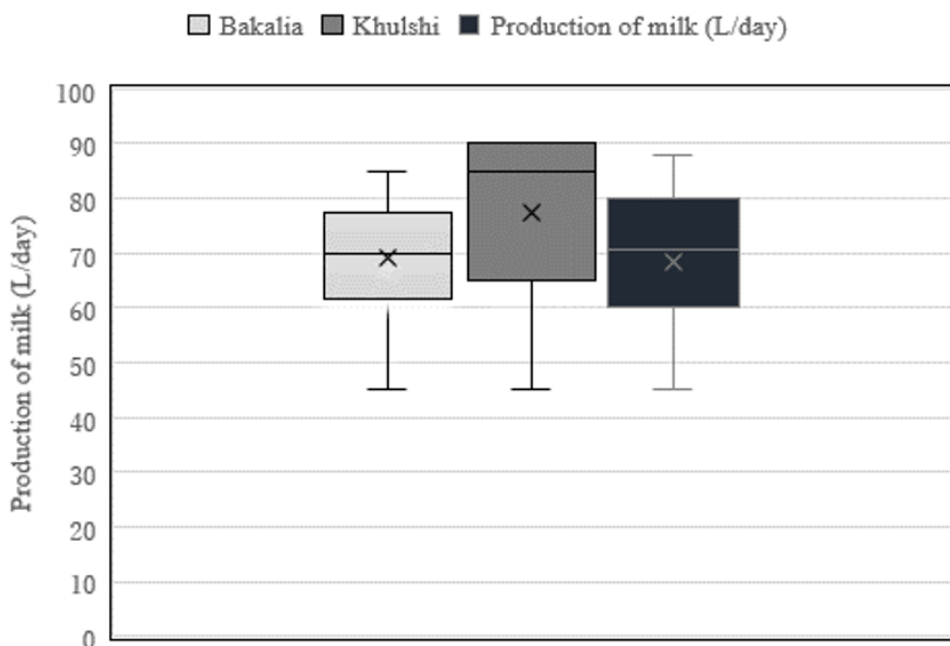

Figure 2. Education level of farm owners

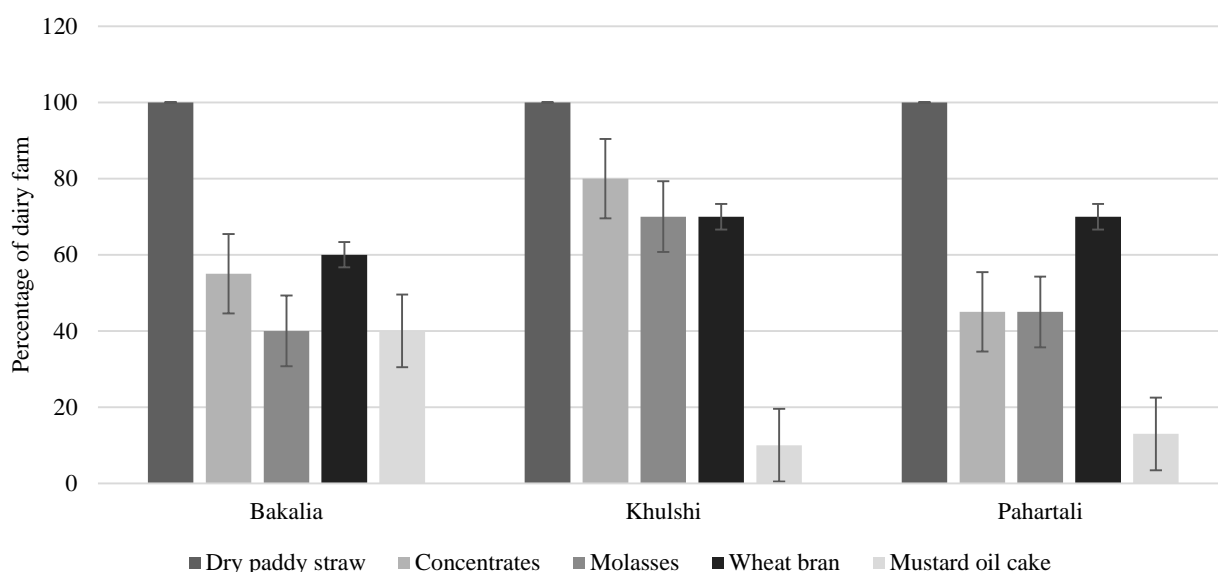Figure 3. Summary of milk production

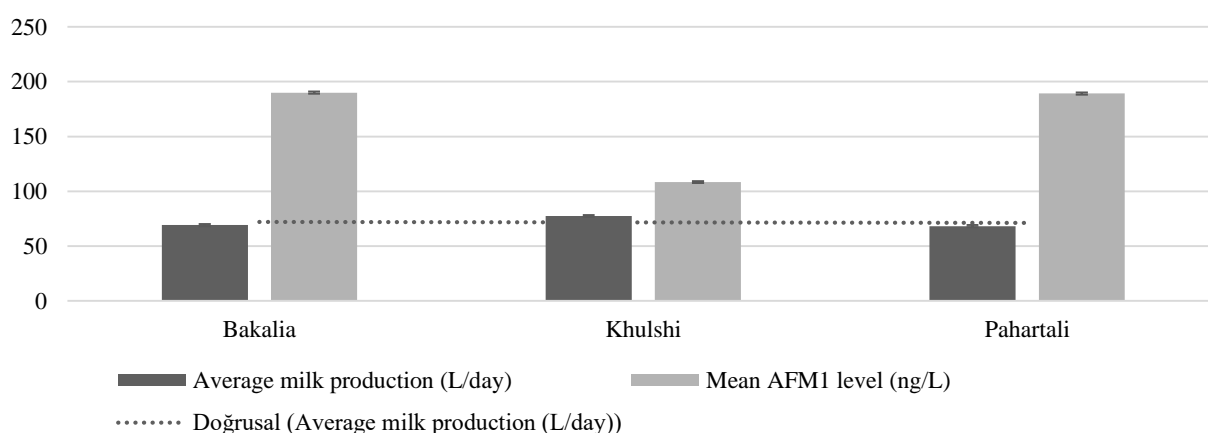Figure 4. Feeding system of different dairy farms

Figure 5. Association between milk production and AFM1 level

Discussion

Milk and other dairy products are always at risk of being contaminated with AFM1. Studies on the prevalence of AFM1 in milk and dairy products have grown both internationally and in Bangladesh in tandem with the rise in milk and dairy product consumption. The AFM1 concentration was measured using the ELISA technique since it has a prompt output and an easy-to-use extraction process with good specificity (Rodriguez Velasco et al., 2003).

The study's findings indicated that approximately 43% of the samples from various dairy farms in Chattogram, Bangladesh, were at least slightly contaminated by AFM1, and the majority of AFM1-positive samples (69%) exceeded the EU's permitted level (50 ng/L) (van Egmond et al., 2007) and few of AFM1- positive samples (5%) exceeded US and BSTI/BFSA regulations (500 ng/L). Two more Bangladeshi researchers supported the findings of the current study (Tarannum et al., 2020; Sumon et al., 2021). In 2020, Tarannum et al. reported that 75% of raw milk samples tested positive for AFM1, with 70% exceeding the EU's permitted limit. Sumon et al. (2021) discovered

71.4% AFM1-positive raw milk samples, whereas 23.8% of samples exceeded the EU limit (50 ng/L). Similar results regarding the presence of AFM1 were discovered between this study result and other studies conducted by Gurses et al. (2004) and Rahimi et al. (2010). However, the outcome of the study carried out by Nemati et al. (2010) is higher (100%) than the outcome attained in the current investigation (43%). AFM1 was found in milk and was linked to concentrates used to feed cattle made of maize germ, cotton, dairy meal, and sunflower seed cake, all of which are susceptible to AFB1 contamination (Makau et al., 2016). The crop production in Bangladesh that is weather-dependent (summer and winter) may be the cause of the predominance of AFM1 in raw milk. Up until the arrival of new crops from a new season, farmers heavily utilize the previous season's yield in the next one (Ali, 2016). Due to prolonged storage times and high humidity levels during the harvest, molds can therefore easily grow on feedstuffs that are creating mycotoxins (Dawlatana et al., 2002). AFM1 contamination in milk is mostly caused by AFB1 contamination of animal feed, and the presence

of AFB1 in forage indicates favorable circumstances for mycotoxin synthesis and mold growth (Norian et al., 2015). Industrial and stored feed is more likely to experience fungal development and the production of aflatoxin, particularly AFB1, which can lead to the presence of AFM1 (Whitlow and Hagler, 2002).

AFM1 is a metabolite of AFB1 that is excreted in milk, hence finding significant amounts of it in raw milk samples implies the presence of extremely high quantities of AFB1 in feed, especially in hay (Elzupir and Elhussein, 2010). As the present study was conducted during the summer and rainy seasons in Bangladesh, the climatic conditions were favorable for mold growth (Roy et al., 2013). Geographic and climatic changes may impact farm management techniques and feed quality (Ghazani, 2009). As a coastal city, with a huge rainfall, the dairy cattle farmer in Chattogram harvests hay in the summer, stores it until the next season, and feeds it to the cattle during the year. This could be the primary cause of the high humidity, high warmth, and improper storage conditions that promote fungal growth and AFB1 in haystacks. While AFB1-contaminated feed is ingested, it is metabolized in the liver, leading to elevated levels of AFM1 excreted in milk (Ghanem and Orfi, 2009). As a result, it's crucial to lessen the prevalence of AFB1 toxins in feedstuffs and take preventative action against conditions that encourage the formation of the toxin. Management practices in harvest and storage regarding the aforementioned factors could decrease AFB1 occurrence in feed as well as AFM1 in milk. Both the creation of efficient detoxification procedures and the prevention of toxin formation in the feed are crucial. To reduce the prevalence of aflatoxin in dairy products in Bangladesh, the government and other food control authorities must implement a food supervision and control system, apply rigorous restrictions, and conduct frequent analytical surveillance.

Conclusions

The majority of the AFM1-positive samples exhibited levels of the toxin over the allowed EU threshold. Humans may develop liver cancer from them. This demands that all efforts be made, including raising awareness, paying attention to feed storage conditions and storage times, as well as maintaining effective surveillance, monitoring, and control over the dairy cattle's entire feeding system. Additionally, the appropriate authority needs to continuously monitor the farm-level maintenance of sanitary feeding conditions for dairy animals.

Conflict of interest

None to declare

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