RESEARCH ARTICLE

Prevalence of Aflatoxin M1 in Milk of Bovine in and around Junagadh, Gujarat State of India

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Abstract

Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 from a contaminated feed. Aflatoxins are toxic, mutagenic and carcinogenic compounds produced as a secondary metabolite by the fungi *Aspergillus flavus* and *A. parasiticus*. The present study was conducted to determine the prevalence of AFM1 in milk by using two different diagnostic methods, *viz*., Enzyme-Linked Immunosorbent Assay (ELISA) and Lateral Flow Assay (LFA). A total of 200 milk samples were collected from the areas under Junagadh city and nearby villages. An overall 93.5% incidence rate of AFM1 contamination in milk samples was recorded. Out of 200 samples, 6 (3%) samples were found to contain AFM1 above the FSSAI standard limit (0.5 µg/L) and 145 (72.5%) samples had AFM1 above the standard limit as per EU legislation (0.05 µg/L). Out of total 200 samples, 50 representative samples analyzed in the LFA, which revealed that the LFA can detect aflatoxin M1 only when level of contamination was above 0.1 µg/L in ELISA.

Keywords: Aflatoxin M1 (AFM1), ELISA, Lateral Flow Assay, Milk.

Ind J Vet Sci and Biotech (2022): 10.21887/ijvsbt.18.3.24

Introduction

Foodborne diseases are caused by pathogens or other food contaminants like toxins and pollutants which are a serious health threat to the human (Anil *et al*., 2020). Mycotoxins are one of the contaminants which are challenging to human health by inducing infection by an allergic reaction or through intoxication. There are about 400 defined mycotoxins produced by the fungal species. Only a few toxins are intoxicated by inhalation; most of the toxins are ingested through various food materials like milk, meat, egg, coffee, cereals, beer, wine, nuts, and many more (Herbert, 2016). Aflatoxin is mostly produced by three fungal species of *Aspergillus, viz*., *A. flavus, A. parasiticus,* and the rare species *A. nomius,* which contaminates the plants and plant-oriented products. *A. flavus* and *A. parasiticus* are common fungi found everywhere showing a particular affinity for oily seeds as a growth source. They colonize plants in the field in a tropical or subtropical and temperate climate, they can also colonize in post-harvested products if the products were not dehydrated adequately (Prandini *et al*., 2007).

Aflatoxins are difuranocoumarin derivatives produced as secondary metabolites through a polyketide pathway by filamentous fungi, mainly by some strains of *A. flavus* and *A. parasiticus*. Aflatoxin is water soluble substance and it is present in milk even after separation of fat or cream (Anil *et al*., 2020). Aflatoxins M1 and M2 are hydroxylated compounds of aflatoxins B1 and B2, respectively, and they are associated with milk due to the ingestion of B1 and B2 aflatoxins contaminated feed (Alex *et al*., 2014). There is a linear relationship between the concentration of aflatoxin M1 in milk and aflatoxin B1 in feed for dairy cattle (Shivraj *et al*., 2015). Due to the toxic effects of aflatoxins, the maximum permissible limit has been set as 0.5 µg/L for milk in India by the Food Safety and Standards Authority of India (FSSAI) (Emmanuel *et al*., 2017). Compared to chromatography method, another rapid and qualitative method such as ELISA is a very popular due to its low cost and easy to use (Zhongming *et al*., 2005). Other than ELISA, commercial lateral flow assay-based (LFA) test is also used to quantify and detect the aflatoxin in milk within a short period of time, like Aflasensor and MRLAFMQ (Wim *et al*., 2014). The present study was aimed to determine the prevalence of AFM1 in bovine milk in Junagadh area by using two different diagnostic methods, *viz*., ELISA and LFA.

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How to cite this article: Hedpara, J.R., Sindhi, S.H., Ghodasara, S.N., Kumar, B., Bhedi, K.R. (2022). Prevalence of Aflatoxin M1 in Milk of Bovine in and around Junagadh, Gujarat State of India. Ind J Vet Sci and Biotech. 18(3), 104-107.

Source of support: Nil

Conflict of interest: None.

Submitted: 12/11/2022 **Accepted:** 29/05/2022 **Published:** 10/07/2022

Materials and Methods

Sample Collection

Milk samples were collected from Junagadh city and nearby villages (Gujarat, India). Samples were labeled with name

of place, date and number for the convenience in analysis of each lot for ELISA test. In each of the selected area, milk samples were collected from individual animal from various farms, milk collection centers and local vendors. All samples were collected in the summer season. A total 200 milk samples were collected which comprised of 106 milk samples from cattle and 94 milk samples from water buffaloes. Fresh milk samples were collected and transported to laboratory with temperature-maintained channel. Samples were stored in deep freezer at -20°C till further analysis. The samples belonged to two categories, *i.e.,* raw milk and pasteurized milk. All the collected milk samples were in duplicate where one sample was used for detection of AFM1 in milk by using ELISA technique while another was used for LFA, if they were found positive in ELISA.

Detection of Aflatoxin M1 in Milk Samples by ELISA

For the ELISA test quantitative analysis of AFM1 in samples was performed using AFM1 test kit (Ridascreen, Aflatoxin M1 Art. No.: R1121, Darmstadt, Germany). The measurement was made photometrically at 450 nm on ELISA reader following the manufacturers' instructions.

Milk samples were chilled at 10°C, of which 2 mL was centrifuged at 3000 x *g* for 10 minutes at 4°C. As aflatoxins are water soluble compounds the upper creamy layers were completely discarded, and the lower phases (skim milk) were used after 10 times dilution for quantitative determination of the concentration of AFM1 as per the FSSAI limit (0.5 µg/L) by ELISA (Nurhan *et al*., 2011).

One-hundred microliter antibody was added to the bottom of each well and incubated for 15 min at room temperature (27°C). The antibody was then poured out and the wells were washed twice with washing buffer (250 µL). One-hundred microliters of standards (0, 5, 10, 20, 40 and 80 ng/L) and 100 µL prepared samples were poured into separate microtiter wells and incubated for 30 min at room temperature (27°C) in the dark. Again, the wells were washed twice with washing buffer. In the next stage, 100 µL of the conjugate was added to the wells, mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Over again, the wells were washed twice with washing buffer. Afterwards, 100 µL of substrate was added in it, mixed gently and incubated in the dark at room temperature for 15 min. Finally, 100 µL of the stop reagent was added to each of the wells. The absorbance value was measured at 450 nm using an ELISA reader within 15 minutes after adding stop solution.

Detection of AFM1 by LFA Method

MRLAFMQ tests (kit lot no.23 (Exp. 06/2021) (Charm Sciences Inc., Lawrence, MA, USA), a 45°C ROSA incubator and Charm EZ reader were used. The MRLAFMQ test for milk is a lateral flow method that works in 8 minutes with a single milk addition step.

In LFA test, samples were prepared by 200 µL of milk sample and 200 µL of sample dilution buffer mixture and this mixture was used for quantitative test. Before using the prepared sample, it was kept in refrigerator for 5 min and then tested (NMSQS, 2018). Once machine was showing insert test strip, lid of machine was opened, and strip was inserted in it and it was automatically detected by machine. Details of the sample were fed in the machine and 300 µL prepared sample mixture was added in the strip. Then lid was closed and after 8 minutes aflatoxin M1 concentration of sample was showed in the display.

Both the test results were compared by using statistical method to find out the best convenient test. Kappa test was used for the comparison of ELISA and LFA test.

Results and Discussion

Distribution of positive and negative milk samples as per FSSAI and EU legislation limit by using ELISA test is shown in Table 1. The result revealed that out of 200 milk samples, 6 (3%) samples were found positive for AFM1 as per the FSSAI limit (0.5 µg/L) and 145 (72.5%) milk samples were found positive (above the permissible limit) as per the European Union (EU Codex) Commission standard limit (0.05 µg/L). Prevalence of AFM1 was found 2.8% (3/106) in case of cattle milk samples and 3.2% (3/94) in buffalo milk samples as per the FSSAI limit. As per EU legislation, 71.6% (76/106) prevalence in cattle milk and 73.4% (69/94) prevalence in buffalo milk samples was observed. Overall, 93.5% (187/200) incidence rate was recorded in this study. Area wise 6.1% prevalence of aflatoxin M1 was found in urban area and 2% in

Table 1: Positive and negative milk samples as per FSSAI and EU MPL by using ELISA

	FSSAI MPL (0.5 µg/L)				*EU legislation (0.05 µg/L)*			
	Positive		*Negative*		*Positive*		*Negative*	
Type of Milk Sample	*C*	*B*	*C*	*B*	*C*	*B*	*C*	*B*
Raw Milk (192)	3	3	100	86	73	65	30	24
Pasteurized Milk (4)	0	0	2	2	2	2	0	0
Boiled Milk (4)	0	0	1	3	1	2	0	1
Total	3	3	103	91	76	69	30	25

C= Cattle; B= Buffalo; FSSAI= Food Safety and Standard Authority of India; EU= European Union; MPL= maximum permissible limit; µg/L= microgram per liter.

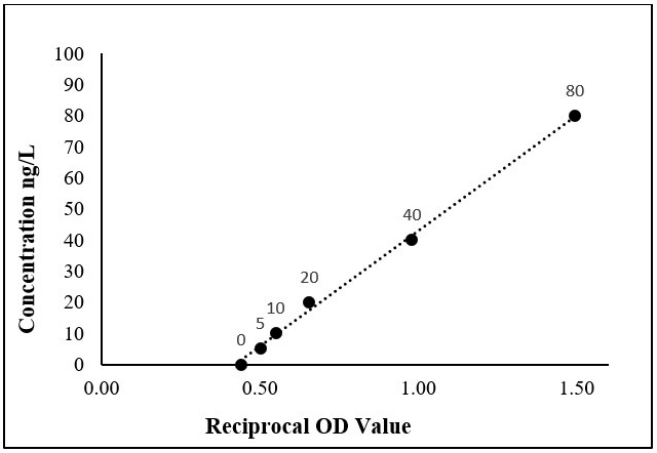

Fig. 1: Standard curve for AFM1 detection by competitive ELISA

rural area as per the FSSAI, and as per the EU legislation 87.8% in urban area and 73.5% in rural area. The standard curve for AFM1 detection by competitive ELISA is given in Fig. 1.

Both ELISA and LFA tests were compared by Kappa test and found 0.465 kappa value, which was in moderate agreement between ELISA and LFA tests as per the kappa scale.

The level of AFM1 in milk samples varied from 0.01 to 0.85 µg/L and the average value of AFM1 for 200 samples was 0.13 µg/L in ELISA. Out of 6 positive raw milk samples, 3 were from cattle and 3 were from buffaloes. We observed 0.85 µg/L the highest concentration of AFM1 and 0.65 µg/L the lowest concentration of aflatoxin M1 in cattle milk sample by ELISA.

Out of 200 milk samples, total 50 milk samples were analyzed in LFA which included 6 positive and 44 negative samples (Plate 1). Out of 44 negative samples 8 milk samples were AFM1 free. As per the result of LFA, overall mean value of 50 samples was 0.13 µg/L, which was similar to the result of ELISA (200 milk samples). Total 21 samples out of 50 representative milk samples had no contamination of AFM1 in it. Results of the LFA test of negative (below MPL) milk samples were inconsistent specially in those samples which had concentration between zero and MPL. During the study, it was observed that the LFA can detect AFM1 only in those samples which were having level of AFM1 above 0.1 µg/L detected earlier by ELISA test.

In the present study, low prevalence rate of AFM1 in milk observed based on FSSAI, may be due to better feed-keeping, hygiene, Proper ventilation and good quality of feed. Good feed keeping practices and ventilated store house prevent the fungal growth which reduce the aflatoxin production in milk. In agreement with the present study, a similar prevalence rate was reported by India National Milk Safety and Quality Survey (NMSQS, 2018). They reported 2.6% prevalence of AFM1 in 456 milk samples as per the FSSAI MPL. Paul *et al*. (1976) recorded higher prevalence (6.2%) of AFM1 from 81 milk samples in Ludhiana as compared to our finding, while comparable result of ELISA test was reported by Fallah (2010).

Plate 1: (a) LFA test display shows actual time to complete the test (b) Negative sample result found in the test (c) Positive sample found in the test along with reading of concentration of AFM1

Byron *et al*. (2020) detected the presence of AFM1 in all (209) raw milk samples by using LFA with the mean value of 0.077 µg/kg in a range of 0.023 to 0.75 µg/L. AFM1 level was exceeded in 71.7% (150/209) samples as per FSSAI limit (0.5 µg/L) which was in contrast to our finding, 0.13 µg/L mean value of total 50 samples. Thirumala *et al*. (2002) also reported comparable concentration of AFM1 in urban area compared to the rural area, while Ali *et al*. (2012) reported similar incidence rate (97.6%) in their study.

Since this study is limited due to the number of samples and area limitations, additional surveillance programs for both feed and milk need to be carried out to be able to perform a risk assessment of AFM1. As a matter of concern to the public health, the high incidence 93.50% of milk samples found contaminated with AFM1 should be viewed with caution. It is pertinent that awareness campaigns are to be conducted for small age group children and adults who are the major consumers of the milk and milk products.

Conclusions

The study concludes that a very high (93.5%, 187/200) incidence rate of AFM1 exists in the raw milk of Junagadh area. Overall, 3% (6/200) prevalence of AFM1 was recorded as per the FSSAI limit, and 72.5% (145/200) based on EU legislation (0.05 µg/L). In buffalo milk the prevalence of AFM1 was 3.2% (3/94) and in cattle milk 2.8% (3/106) as per the FSSAI limit. The current results imply that more emphasis should be given to the regulation of AFM1 levels in milk. Comparison of LFA with ELISA for detectability of AFM1 shows moderate agreement in kappa test. ELISA is superior in terms of determination of the level of AFM1 in milk samples due to its consistent results in positive and negative milk samples.

Acknowledgement

Authors are grateful to the authorities of Kamdhenu University, and the Dean, College of Veterinary Science & AH, KU, Junagadh for the support and facilities provided.

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ANNOUNCEMENT: SVSBT-NS-2022

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