REPORT

Occurrence of Aflatoxin M_1 in raw and processed milk consumed in Pakistan

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Abstract: Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of Aflatoxin B_1 (AFB_1). It appears in milk, when lactating animals consume AFB_1 contaminated feed. It is carcinogenic and teratogenic in nature. Present study was planned to determine levels of AFM_1 in raw and processed milk. For this, a total of five hundred and seventy milk samples (raw = 340 and processed = 230) were collected from Punjab (province of Pakistan). Processed milk included ultra-heat treated (UHT) (n=105), pasteurized (n=65), dried (n=40) and condensed milk (n=20). Concentration of AFM_1 was quantified by direct competitive ELISA technique. Analysis revealed 100 percent incidence of AFM_1 in UHT and pasteurized milk with a mean of 0.35±0.28ng/ml and 0.11±0.03ng/ml respectively. However, 86.66% raw milk samples were tainted with AFM_1 with mean of 0.52±0.42ng/ml and 66.66% of dried milk samples with mean of 0.03±0.02ng/ml. However, none of the condensed milk sample was found positive. Data of raw milk contamination was further computed for seasonal variation. Highest prevalence (100%) was observed during autumn season followed by winter (81.81%), summer (80%) and spring season (62.06%) respectively. Furthermore, all mean values except raw milk were below the FDA legislation. Study results indicate the possible adverse effects on health of people of Pakistan. Good agriculture practices (GAP) and regular screening of raw materials of animal feed prior to supplying may help to control AFM_1 levels in milk.

Keywords: Aflatoxins, milk, season, feed, legislation.

INTRODUCTION

Worldwide contamination of food and feed with mycotoxins is a significant problem. Mycotoxins are toxic secondary metabolites of filamentous fungi produced in optimal environmental conditions. Aflatoxins, a group of toxic coumarin rings are produced mainly by four different species *of Aspergillus (A)* like *A. flavus, A. parasiticus, A. ochraceous and A. nomius.* Aflatoxins (AFs) are well studied mycotoxins worldwide. The most important aflatoxins in order of toxicity are $AFB_I > AFB_2 > AFG_1 > AFG_2$ respectively. AFB_1 is highly potent in nature and classified as class 1A carcinogen (IARC, 1993). Aflatoxin B_1 (AFB_1) is considered as most potent natural mycotoxin due to its carry over effect from animal feed to human food. Hence, it is a matter of global concern over food and feed safety (Langat *et al*., 2016).

Aflatoxin M_1 is 4-hydroxy metabolite produced in liver as a result of biotransformation of AFB_1 by cytochrome P_{450} enzymes.It appears in biological fluids (i.e. Cerebrospinal fluid, urine, serum, milk etc). AFM_1 is also excreted in milk of both human and lactating animals that have been fed with AFB_1 contaminated diet (Fallah *et al.,* 2010). The conversion of AFB_1 into AFM_1 varies with animal breed, health, mammary infection, milking time, lactation stage, season, ingested levels and duration of exposure to AFB_1 contaminated feed (Asi *et al*., 2012; Durate *et al*., 2013). Generally, AFM_1 appears in milk after 8-12 hours of AFB_1 contaminated feed ingested at a rate of 0.3-11% (Karaimi *et al.,* 2007; Britzi *et al.,* 2013). Several studies showed that AFM_1 is comparatively stable at high temperature and acidic treatment during processing of milk and its products. It may be reduced but not completely destroyed by heat treatments such as pasteurization, UHT technique and autoclaving (Motawee *et al.*, 2004; Tavakoli *et al*., 2013). Therefore, appearance of AFM_1 is obvious in processed milk and milk products, if raw milk is contaminated (Duarte *et al*., 2013).

Consumption of contaminated milk and milk products is the principal route of AFM_1 entry into human body. It may cause serious human diseases i.e. primary liver cancer, hepatic cirrhosis, hepatitis, DNA damage, gene mutation and chromosomal anomalies (Kos *et al.*, 2014; Motagna *et al.*, 2008). It is also classified as a group 1A human carcinogen by international agency of research on cancer (Creppy, 2002). Due to injurious health effects of AFM_1, various countries have established their regulatory limits

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for AFM_1 ranging from 0.02 to 0.5ng/ml in milk according to their surveillance studies (Karaimi *et al*., 2007). However, internationally, most commonly adopted permissible limits for AFM_1 are 0.05ng/ml and 0.5ng/ml by European Commission (EC, 2006) and Food and Drug Administration (FDA, 2011) respectively. In Pakistan, there is no regulatory limit defined for AFM_1 in milk.

Milk, as an essential component of daily diet providing all basic nutrients required during an individual's life span (Paniel *et al.*, 2010). It is mainly consumed by most vulnerable age groups because of their limited food choices. Hence, screening of milk for AFM_1 contamination round the year is also imperative. In Pakistan, some studies reported AFM_1 contamination in raw milk samples with emphasis on animal breed and seasonal variations (Asi *et al*., 2012; Ismail *et al.*, 2015). However, level of AFM_1 contamination in processed milk has not been studied so far. In view of this background, present study was planned to evaluate the contamination level of AFM_1 in raw and processed milk (i.e. ultra heat treated (UHT), pasteurized, and dried and condensed milk). Moreover, seasonal variation of AFM_1contamination in raw milk was also recorded during this study.

MATERIALS AND METHODS

Sampling

A total of five hundred and seventy milk samples i.e. raw (n=340) and processed (n=230) were collected from dairy farms, retailers and markets of Punjab during October 2013 to December 2015. Processed milk samples included UHT (n=105), pasteurized (n=65), dried (n=40) and condensed milk (n=20). The samples were labeled and preserved at -20°C till further analysis.

Quantitative Analysis of AFM_1 by ELISA

Milk samples were analyzed according to the instructions of ELISA kit (Agra Quant® Aflatoxin M_1 sensitive catalogue # COKAQ7100) provided by manufacturer (Romer,Singapore). Kit contained dilution wells, antibody coated wells, washing buffer, standards (i.e. 0, 25, 50, 100, 200 and 500ng/L) conjugate, substrate and stop solution. All reagents were brought at room temperature prior to analysis.

Sample preparation

For sample preparation, 5ml of liquid milk (i.e. raw, UHT and pasteurized) was placed at 4°C for 30 minutes. For dried and condensed milk, 10g of each milk sample was added to 100ml of deionized water and homogenized by using magnetic stirrer at 40°C for 25 minutes. Samples were then centrifuged at 3000 ×g for 10 minutes. After this 0.4ml of milk (below fat) was mixed with 0.1ml of methanol.

AFM_1 determination

Initially, 200µl of conjugate solution was added in dilution wells already placed in microtitre plate. Then 100 µl of standard solutions (i.e. 0, 25, 50, 100, 200 and 500 ng/L) and prepared samples (i.e. raw, UHT, pasteurized, dried and condensed) milk were added into dilution wells. These solutions were mixed by up and down pipetting five times to avoid any cross contamination. After mixing, 100µl of this solution was transferred to antibody coated micro wells already placed in microtitre plate and incubated for 60 minutes at room temperature (25°C) in dark. On completion of incubation, wells were drained into waste container and washed three to five times by using washing buffer. Wells with strips were then tapped on paper towel to remove as much water as possible. In next step, 100µl of substrate (horseshoe peroxidase) was added into all microwells, mixed gently and incubated for 20 minutes under dark conditions again. Finally, 100µl of the stop solution (1N H_2SO_4) was added into the microwells and colour changed from blue to yellow. The absorbance data of samples were recorded at 450 nm by using BioTek ®ELISA Reader Elx808 (BioTek®, USA). The OD data was computed to concentration by using BioTek® Gen5 software (BioTek®, USA). Limit of detection of method was 0.01ng/ml for liquid milk whereas 0.02ng/ml for dried and condensed milk.

STATISTICAL ANALYSIS

Data was subjected to SPSS-16 software for statistical analysis. One-way analysis of variance followed by Duncan multiple test was applied to determine the significant difference ($p<0.05$) between different seasons and all milk types.

RESULTS

AFM_1 contamination Levels

Results of AFM_1 contamination in milk samples are summarized in table 1. Present study revealed, a significant ($p<0.05$) low mean levels observed in UHT (0.35±0.28ng/ml), pasteurized (0.11±0.03ng/ml) and dried milk (0.30±0.02ng/ml) as compared to raw milk samples (0.52±0.42ng/ml). Condensed milk samples were found free of AFM_1 contamination.

Seasonal distribution of AFM_1 contamination

Environmental factors i.e. temperature, rainfall, humidity etc. affects AFB_1 contamination in animal feed and as consequence AFM_1 concentration in milk varies with seasons. Therefore, present results of AFM_1 concentration in raw milk samples were further computed for seasonal variation Results showed, a significant ($p<0.05$) high mean level was observed in winter (0.46± 0.38ng/ml) and autumn (0.40±0.29ng/ml) as compared to summer 0.21±0.16ng/ml and spring 0.12±0.27ng/ml (table 2).

Table 1: Natural incidence (Mean ±SD) of Aflatoxin M_1 in raw and processed milk samples with reference to FDA legislation

Milk Type		Sample (n)	Positive (%)	Mean ± SD (ng/ml)	Range (ng/ml)	<FDA (%)	>FDA (%)
Raw Milk		340	86.66	0.52^{c}± 0.42	0.17-1.63	65.55	34.45
Processed	UHT Milk	105	100	0.35^{b}± 0.28	0.01-0.95	83.34	16.66
	Pasteurized Milk	65	100	0.11^{a}± 0.03	0.07-0.15	100	Zero
	Dried Milk	40	66.66	0.03^{a} ±0.02	0.01-0.08	100	Zero
	Condensed Milk	20	Zero	$<0.01^{a}$	BDL*	100	Zero

*BDL- Below Detectable Limit
[a-c]Mean with different superscript differs significantly (P<0.05)

Table 2: Seasonal variation (Mean ± SD) of AFM_1 contamination in raw milk samples

Seasons	Positive (%)	Mean ± SD (ng/ml)	Range (ng/ml) (Mini. - Max.)	Feeding Practices
Autumn (Sept, Oct, Nov)	100	0.40^{b}±0.29	0.01-1.09	No Fresh Feed i.e. Preserved Hay, silage, cotton seed cake
Winter (Dec, Jan, Feb)	81.81	0.46^{b}± 0.38	0.01-1.63	
Spring (Mar, Apr, May)	62.06	0.12^{a}±0.27	0.01-0.25	Fresh Feed i.e., green fodder, grazing, weeds
Summer (June, July, Aug)	80.0	0.21^{a}±0.16	0.01-0.97	

[a-b]Mean ±SD with different superscript differs significantly (p<0.05).

DISCUSSION

Worldwide milk and milk products (i.e. ice cream, yogurt, cream, cheese etc.) are being used as a nutritional diet. Therefore, AFM_1 contamination in milk is a problem of global concern. According to present results, significant (p<0.05) low mean levels of AFM_1 were recorded in processed milk samples. It may because of the fact, the processed milk subjected to high temperature treatments (i.e. UHT, pasteurizing, autoclaving etc.) to kill harmful microbes and to increase the shelf life of milk. Some studies reported that high temperature treatments reduce AFM_1 upto 40%. (Fallah *et al.,* 2010; Iha *et al*., 2013). During the process of screening, if levels of AFM_1 are below than FDA regulatory limits i.e. 0.5ng/ml, only then the milk is selected for further processing methods i.e. UHT, Pasteurized, condensed and dried milk. This selection of milk might be the reason for low mean levels in processed milk samples during present study. Moreover, dilution of milk with water or defatting to maintain a specific level of fat i.e. 3.5% (Battocone *et al.*, 2005) might be another contributing factor for significant (p<0.05) reduced mean levels in processed milk samples as compared to raw milk samples.

As far as percentage incidence was concerned, highest percentage of prevalence was observed in UHT and pasteurized (100%) followed by raw (86.66%) and dried (66.67%) milk samples. These results confirm the heat stable nature of AFM_1. It can further be explained on the basis of melting point of AFM_1 i.e. 228°C (Sanli *et al*., 2012). Ultra heat treated milk is processed at 140°C for 2 seconds and pasteurized milk is heated at 72°C for 15 seconds (Fallah *et al.*, 2010; Sanli *et al*., 2012; Duarte *et al.,* 2013). Such heat treatments may reduce AFM_1 (upto 40%) but cannot eliminate it completely. Albeit, no data available from Pakistan to compare regarding AFM_1 contamination in processed milk i.e. UHT, pasteurized and dried milk. However, observed mean levels of processed milk were similar as reported in Iran (pasteurized; 0.23ng/ml; dried, 0.07ng/ml) by Karaimi *et al.* (2007); Kamkar *et al*.,(2011) respectively. A study conducted in Brazil also documented similar results (UHT; 0.11ng/ml) by Shudno and Sabino (2006). Similarly, present findings are line with study conducted in China where 96.2% pasteurized samples were reported positive with a range of 0.023-0.154ng/ml (Zeng *et al*., 2013). Interestingly, present study revealed that condensed milk samples were found free of AFM_1. All condensed milk samples included in present study were imported brands of European origin. It may be due to the strict regulations and monitoring regarding AFM_1 contamination in Europe. To minimize the human exposure, different countries have been defined regulatory limits according to their surveillance studies. These permissible levels vary from 0.02 to 0.50ng/ml). However, internationally most commonly adopted permissible limits for AFM_1 are 0.5ng/ml (FDA, 2011) and 0.05ppb (EC, 2006). Presently, permissible limit (0.5ng/ml) defined by FDA is being followed in Pakistan. Therefore, results of present study were compared with FDA legislation (2011) as can be seen in table 1. As far as raw milk is concerned, in present study comparatively less samples (34.45%) exceeded FDA regulation as compared to previously reported (99.4%) in Pakistan by Hussain *et al.* (2008). It might be due to awareness and improved managemental practices to control AFB_1 at farms level. Moreover, these days, soybean and canola

meals have been replaced with corn and cotton seed cake (which are considered as favorable substrates for AFB_1 production) in animal feed. In addition to this, inoculants used for silage making also have antifungal activity. Phenyl lactic acid is integral component of inoculants. Such chemical compounds are also reported as helping agents to reduce AFB_1 contamination because of its fungal inhibitory action (Mandal *et al.*, 2007). Moreover, only 16.66% positive samples of UHT milk exceeded FDA legislation i.e. 0.50ng/ml, whereas, none of the pasteurized dried and condensed milk samples exceeded the FDA regulation (table 1).

As far as raw milk is concerned, a significantly ($p<0.05$) higher mean level of 0.52±0.42ng/ml was observed. There are many factors responsible for high mean levels in raw milk samples as compared to processed milk. Firstly, these significant ($p<0.05$) high mean levels of AFM_1 were an indication of higher AFB_1 in ingested feed. Infact, small farmers generally choose cheaper feed sources (stale bread, cotton seed cakes, mustard cake etc.). These ingredients are low-priced but highly susceptible for fungal growth and mycotoxin production. Therefore, such inappropriate selections of feed ingredients lead to high AFB_1 contamination in feed. In addition to other factors, a common practice of using bakery waste also enhances the level of AFB_1 and resulting in high AFM_1 contamination in milk. Moreover, raw milk does not go any further high heat or preservation treatments and thus, may lead to comparatively higher mean levels. Secondly, raw milk is usually collected and pooled without any prior screening for AFM_1 contamination. Moreover, sample collection time is also a contributing factor for this variation in results. Mean levels of the present study (0.52ng/ml) was not in line with the previously reported (i.e. 0.13ng/ml) in raw milk by Hussain *et al.* (2008) and 0.38ng/ml by Jawaid *et al*. (2015). This variation among results may be due to low number of samples (n=84) in previous studies as compared to the present study (n=320). Moreover, these studies were area specific

In Pakistan, a survey revealed that processed milk is consumed by only 14% of population, while 70% populatio prefer to use raw milk (Gallup and Gillani, 2011). Furthermore, different types of processed milk (i.e. dried, UHT, pasteurized and condensed milk) are prepared from raw milk after different treatments like evaporation, heating etc. Therefore, raw milk contamination is most important to monitor throughout the year. As AFM_1 residue level in milk is directly proportion to AFB_1 contamination in animal feed.Afb1 contamination in feed is greatly affected by seasons and environmental factors (i.e. drought, stress, insect infestation, humidity temperature).

In the present study, percentage incidence of AFM_1 during four seasons was: winter>autumn>summer>spring table 2. These significant higher levels of AFM_1 in autumn and winter seasons are a consequence of higher AFB_1 contamination in animal feed during these seasons. In fact, limited fresh feed i.e. grass, weeds and pasture is available to animals during autumn and winter seasons. Therefore, animals are mainly fed on stored feed comprising corn, cotton seed, mustard cake, silage and stored hay etc. (Asi *et al.*, 2012). In addition, kitchen wastes are commonly used in combination with animal feed in winter. Generally, these left overs are heavily loaded with fungal contamination because of their carbohydrates contents and hygroscopic nature (Ismail *et al*., 2015). The most determining factors for the production of AFB_1 are temperature, moisture and humidity (Mohammad *et al*., 2010). Aflatoxigenic fungi i.e. *A. flavus* and *A. paracitcus* can produce AFB_1 at around 25°C with relative humidity is >70%. In Pakistan, temperature range during autumn is 13-25°C; relative humidity is >50 and in winter it is between 2°C to 12°C and relative humidity of >70%. In addition to these favorable environmental factors poor storage conditions also induce AFB_1 production which in turn responsible for high levels of AFM_1 in milk in winter and autumn (Kamkar *et al*., 2011, Ismail *et al*., 2015).

CONCLUSION

In the current study, comparatively low mean levels of AFM_1 were found in processed milk as compared to raw milk. However, even low levels of AFM_1 in milk remain health hazard particularly for infants. AFM_1 transmission in milk can be reduced only by controlling AFB_1 contamination in animal feed and feed ingredients. This can be achieved by adopting Good Agriculture Practices (GAP) at farm level as well as improved storage conditions. Furthermore farmers, farm managers and all stakeholders of dairy industry should be educated for the potential deleterious effects of AFM_1 on human health. Electronic and print media may play important role in this regard. Above all, it is important to set regulatory limits by government to save the population. Present study constitutes first ever report regarding processed milk in Pakistan. This study provides a base to evaluate the daily intake of AFM_1 by using milk and milk products i.e. cheese, yogurt, ice cream, butter etc. Such studies on regular basis should be conducted at national level. These studies will be helpful to control the health risk factors and supply of AFM_1 free milk to our population.

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