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Aflatoxin M1 in raw milk in summer season in Lahore, Pakistan

N. Zahra[1]*, M. K. Saeed[1], Z. Ahmed[2], M. U. Zain[2] and T. Jalal[2]

[1]*Food and Biotechnology Research Centre, PCSIR Laboratories Complex, Lahore, Pakistan*
[2]*COMSATS University Islamabad, Islamabad, Pakistan*

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Abstract

A four-month long study throughout the summer of 2022 i.e., April, May, June, and July; was conducted to determine Aflatoxin M1 concentration in raw milk retailed in the shops of Lahore, Pakistan. A total of 40 samples (10 per month) were analyzed using the ELISA kit method while considering European Union (50ng/kg), US Food and Drug Regulation Authority (500ng/kg), and Pakistan Pure Food Regulations (500ng/kg) as AFM1 permissible limits. About 82.50% of 40 samples were contaminated with AFM1 while only 17.50% samples were found to be uncontaminated. Of these contaminated samples over 87.88% samples exceeded the safe limit as set by the European Union i.e., 50ng/kg with only 12.12% of the samples being safe for use. On the other hand, only 6.06% of the samples were unfit as per the USFDA & Punjab Pure Food Regulations (PPFR) limit i.e., 500ng/kg. A mean value of 133.57 ± 0.14ng/kg of AFM1 contamination was calculated ranging from 5.43 ± 0.05ng/kg to 964.75 ± 0.04ng/kg in April and July, respectively. The lowest average contamination was recorded in May i.e., 68.35 ± 0.258ng/kg which went progressively higher in the later months due to unusual record-breaking rainfall in the region while highest average contamination was found in the month of July i.e., 228.789 ± 0.075ng/kg. The current study is significantly important as it identify the contamination of Aflatoxin M1 in raw milk in the summer season.

Keywords: Mycotoxins; Aflatoxin M1; Raw Milk; Contamination; ELISA; Summer

Introduction

Aflatoxins are the carcinogenic metabolites of fungi produced by various species of Aspergillus i.e., *A. flavus* and *A. parasticus* that can cause contamination in derivatives and products of plants (Creppy, 2002). The two species of mold infect cereals and oil seeds that are majorly consumed as cattle feed. Their growth is influenced by numerous factors like temperature, relative humidity, oxygen availability, and damaged or broken grain kernels (Awasthi *et al.* 2012).

There are different classes of Aflatoxins include (AFB1-aflatoxin B1), (AFB2- aflatoxin B2), (AFG1- aflatoxin G1) and (AFG2- aflatoxin G2). Due to high toxicity, teratogenicity, hepatocarcinogenicity and mutagenicity; the International Agency for Research on Cancer has categorized aflatoxins putting AFB1 under "Group I" (Iqbal *et al.* 2011; Ostry *et al.* 2017). The cattle consuming a fungal-infested feed may ingest Aflatoxin B1 with it. This Aflatoxin B1 is then hydroxylated to Aflatoxin M1 by the enzyme Cytochrome P450 inside its liver. This hydroxylated Aflatoxin M1 is then excreted in the milk of that cattle making it unfit for use (Forrester *et al.* 1990).

Limited research data is available from the past regarding the health effects of Aflatoxin M1. This is because it is very hard to isolate a large quantity of Aflatoxin M1 in pure form to conduct extensive toxicological research for this compound (Eaton and Groopman, 2013). However, it has been confirmed that Aflatoxin M1 is comparatively less toxic than Aflatoxin B1 and the sequence of their toxicity is given as AFB1+AFM1 > AFB1 > AFM1 (Li *et al.* 2018).

*Corresponding author's e-mail: drnaseemzahra@gmail.com

The toxicological effects of Aflatoxin M1 that have been reported to date include carcinogenic effects (Cullen *et al.* 1987), oxidative stress on Kidney (Li *et al.* 2018), and several immunosuppressive effects (Luongo *et al.* 2014). The combined effect of AFM1 and AFB1 working synergistically with Hepatitis B virus and causing a 12 fold rise in liver cancer risk have been reported as well (Sun *et al.* 2013).

There are various regulatory limits for Aflatoxin M1 contamination in liquid raw milk throughout the world depending upon the economic conditions and availability of resources in the region (Stoloff *et al.* 1991; Van Egmond, 1989). As per the standards of the European Union, the maximum limit allowed for AFM1 contamination in liquid raw milk is 50ng/kg (European Commission, 2010). The limit of 500ng/kg has been allowed by US Food and Drug Administration, and Punjab Pure Food Regulations, Pakistan (US Food and Drug Administration, 2000; Punjab Pure Food Regulations, 2018) Hence, there are varying differences in the maximum permissible limit of AFM1 in liquid raw milk among different countries and regions of the world (Egmond, 1989).

Data from previous studies conducted on Aflatoxin M1 Milk contamination showed varying contamination levels in different regions of Pakistan. A previous study from year 2011 conducted in various regions of Punjab province reported 64ng/kg as the mean AFM1 contamination level in milk from urban areas, with over 42% and 15% samples exceeding the European Union (i.e., 50ng/kg) and USFDA (i.e., 500ng/kg) limit, respectively (Iqbal *et al.* 2014). The same study showed 40ng/kg as the mean AFM1 contamination level in milk from rural farmhouses with over 27% and 8% samples exceeding the European Union and USFDA limit, respectively (Iqbal *et al.* 2014). On the contrary, a study from Lahore in 2007 reported 17.380ng/kg as the mean AFM1 contamination level in milk, with over 81% of samples exceeding the European Union limit (Muhammad *et al.* 2010). Another longitudinal one year-long study from 2018 reported a concentration of 1535ng/kg as the mean AFM1 contamination level in raw milk of Islamabad, with over 91.9% of samples exceeding the European Union limit (Yunus *et al.* 2019). These varying differences in the results could be due to varying temperature conditions, different seasons, feed, and storage conditions used by the farmers in different areas and also differences in the approach for quantification of AFM1 by the researchers. Such studies can therefore help to determine the factors responsible for varying levels of AFM1 contamination in different regions which allows taking region-specific measures to control further contamination in the future.

The present 4 months' study was also designed to assess AFM1 contamination levels in raw milk from different regions of Lahore throughout summer in compliance with the International and National regulatory limits using ELISA technique.

Materials and methods

Sampling

A random collection of Raw Milk samples was conducted from multiple regions of Lahore. The process of sampling continued throughout the months of summer, 2022 i.e., April, May, June, and July. Each month 10 different samples of 500ml raw milk were collected from different retail shops in a pre-sterilized glass bottle and then transported in ice-packed coolers to the Aflatoxin Testing Lab at PCSIR Laboratories Complex, Lahore. It was ensured that fresh samples were collected each time. The samples were kept at a temperature of -20^{o}C before being analyzed for AFM1 detection (Muhammad *et al.* 2010) and their identity was also masked from analysts to keep the study blind and unbiased.

Aflatoxin M1 analysis

The samples of raw milk were analyzed for AFM1 contamination using the ELISA Kit (8019 Veratox® for Aflatoxin M1, NEOGEN, USA) following the protocols as mentioned by the manufacturer. The limit of detection (LOD) and range of quantitation of the kit used was 4.3ng/kg and 5-100ng/kg AFM1, respectively. Samples lying outside this range were quantitated by dilution instructions as provided by the manufacturer.

Before analysis, the kit was validated to avoid any errors in the readings during the study. To initialize the test, milk samples were first incubated at 4^{o}C for 30-35 min. Then a subsample of 5ml from each sample is transferred to test tubes and centrifuged at 3000 rpm for 15 min. The obtained serum was then used to run the test while discarding the fat layer beforehand. Furthermore, the test was performed as specified by the kit manufacturer. Additionally, 6 standards as provided by the manufacturer were also run with each new batch of test to ensure its validity.

After completion of the test procedure, all the microwells carrying subsamples and 6 standards were analyzed using Neogen® Stat-Fax 4700 Microwell Reader for quantitation.

Results and discussion

The levels of Aflatoxin M1 contamination found in raw milk samples collected throughout the summer are given in

Table I. These levels ranged from 5.43 ± 0.05ng/kg to 964.75 ± 0.04ng/kg with an average concentration of 133.5695 ± 0.14ng/kg. Analysis showed that 82.5% of 40 samples were contaminated with Aflatoxin M1. These results can be seen in Table II. Previously, a study revealed 100% AFM1 contamination in 40 samples of raw milk in Lahore (Zahra *et al.* 2020). Similarly, a high prevalence of 86.66% AFM1 contamination in 340 samples has been reported in Punjab as well (Tahira *et al.* 2019). Contrary to this, a quite low percentage of AFM1 prevalence i.e., 47.5% and 51.5% was found in raw milk from regions of Punjab and NWFP, respectively (Iqbal *et al.* 2011).

Universally, the most commonly followed AFM1 permissible limits include the limit of the European Union i.e., 50ng/kg and Food and Drug Regulation Authority legislation i.e., 500ng/kg. (European Commission, 2010; US Food and Drug Administration, 2000). The limit set by the FDA (500ng/kg) is currently being officially followed in Pakistan under the Punjab Pure Food Regulations (Punjab Pure Food Regulations, 2018). Therefore, the results of the present study were analyzed considering both the permissible limits i.e., EU (50ng/kg) and FDA (500ng/kg) as shown in Tables I and IV. Analysis showed that 87.88% of the contaminated samples exceeded the European Union limit, including 6.06% of samples even exceeding the limit of the FDA and PPFR as well. A graphical representation of this

Table I. Quantity of Aflatoxin M1 from April-July

Month	Sr. No.	MQ. ± SD (ng/kg)	Month	Sr. No.	MQ. ± SD (ng/kg)	Total Avg. (ng/kg)	SD. (ng/kg)
April	1	5.43 ± 0.05	June	21	378.28 ± 0.51	133.57	0.14
	2	91.38 ± 0.18		22	351.3 ± 0.09		
	3	100.49 ± 0.10		23	68.12 ± 0.17		
	4	125.35 ± 0.03		24	87.75 ± 0.11		
	5	119.32 ± 0.12		25	0.00		
	6	0.00		26	124.69 ± 0.08		
	7	92.43 ± 0.11		27	325.55 ± 0.36		
	8	98.39 ± 0.08		28	226.65 ± 0.04		
	9	0.00		29	65.28 ± 0.08		
	10	51.25 ± 0.05		30	59.72 ± 0.03		
May	11	82.97 ± 0.89	July	31	44.24 ± 0.06		
	12	0.00		32	107.73 ± 0.13		
	13	263.83 ± 0.04		33	67.97 ± 0.33		
	14	63.23 ± 0.51		34	36.79 ± 0.02		
	15	0.00		35	421.36 ± 0.07		
	16	20.2 ± 0.37		36	964.75 ± 0.04		
	17	93.93 ± 0.18		37	0.00		
	18	96.12 ± 0.40		38	518.63 ± 0.05		
	19	0.00		39	70.02 ± 0.01		
	20	63.23 ± 0.19		40	56.4 ± 0.04		

- MQ. Stands for mean quantity calculated in parts per trillion *(ng/kg)*.
- SD. Stands for Standard Deviation calculated in parts per trillion *(ng/kg)*.

Table II. Statistical Results of Aflatoxin M1 Contamination

Month	Samples tested	Contaminated			Uncontaminated		
		No. of contaminated samples	Total	Percentage	No. of uncontaminated samples	Total	Percentage
April	10	8	33	82.50%	2	7	17.50%
May	10	7			3		
June	10	9			1		
July	10	9			1		

Table III. Contaminated samples exceeding permissible limits

Month	Samples tested	European Union (>50 ng/kg)			FDA/PPF R (>500ng/kg)		
		No. of unfit samples	Total	Percentage	No. of unfit samples	Total	Percentage
April	10	7	29	87.88%	0	2	6.06%
May	10	6			0		
June	10	9			0		
July	10	7			2		

can also be seen in Figure 1 and 2. Similar results have already been reported in another study conducted in Lahore in Spring and Summer, where assessment of 94 raw milk samples revealed 71% of samples exceeding the EU limit while only 6.3% of samples exceeding the limit of the FDA and the PPFR (Ahmad *et al.* 2019). Another study conducted throughout the year 2015 on a total of 240 samples from different regions of Punjab showed 53% of samples exceeding the FDA limit (Akbar *et al.* 2020).

The mean quantity of AFM1 contamination in each month from April-July is given in Table V. The outcomes showed average concentrations of 68.404 ± 0.0723ng/kg, 68.351 ± 0.258ng/kg, 168.734 ± 0.147ng/kg and 228.789 ± 0.075ng/kg in April, May, June, and July, respectively (Table V).

The highest mean quantity of AFM1 can be seen in July, while the lowest was found in May, although the mean quantity of each month lay within the limit of the FDA and the PPFR but exceeded the EU limit. The pattern of change in AFM1 contamination with each month throughout summer can be depicted from the slope of Figure III. The ideal growth conditions for species of fungi like A. flavus and A. parasiticus contaminating the feed are: 13-18% feed moisture while environmental humidity range between 50% to 60%.

Table IV. Contaminated samples within permissible limits

Month	Samples tes ted	European Union (<50 ng/kg)			FDA/PPFR (<500 ng/kg)		
		No. of fit samples	Total	Percentage	No. of fit samples	Total	Percentage
April	10	1	4	12.12%	8	31	93.94%
May	10	1			7		
June	10	0			9		
July	10	2			7		

Table V. Variation of Aflatoxin M1 contamination throughout the summer

Month	Range (ng/kg)	MQ. (ng/kg)	SD. (ng/kg)
April	5.43-125.35	68.404	0.0723
May	20.2-263.83	68.351	0.258
June	59.72-378.28	168.734	0.147
July	36.79-964.75	228.789	0.075

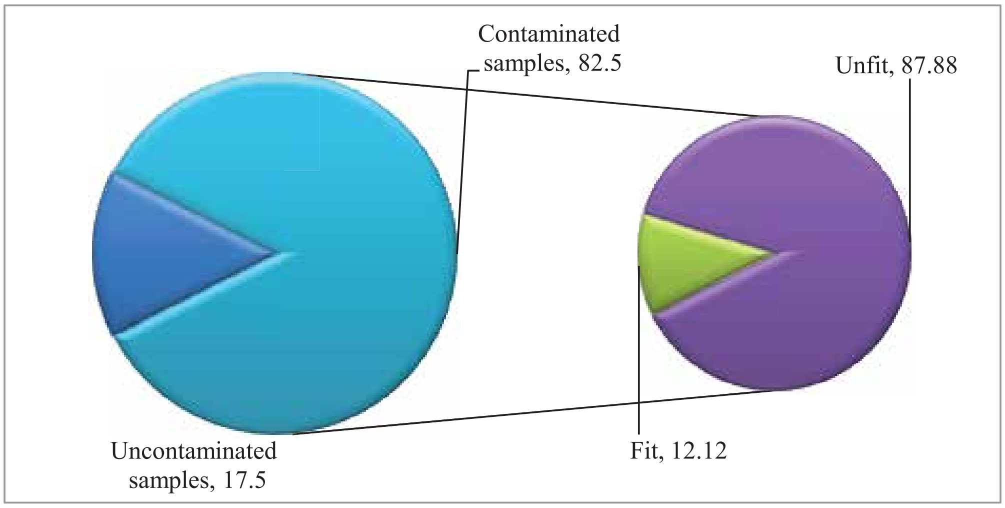

Fig. 1. European union sample analysis

The mold of the specie then requires a Temperature of 25°C and a relative humidity range of 85-90% to produce the toxin, AFB1 (Bakirci, 2001). This may be the reason for the sudden hike in AFM1 contamination in June which then prevailed in July as well. Punjab and so is Lahore experienced record-breaking rain i.e., 62.1mm/+110.9% and 224.3mm/+115.5 during June and July, respectively (Pakistan Meteorological Department, 2022; Pakistan Meteorological Department, 2022). Hence, contributing to a more humid atmosphere and providing ideal conditions for Fungi to grow. The pattern of results is also following various contemporary studies in which high production of Aspergilus species, the primary source of AFB1, was reported in more humid conditions (Lević *et al.* 2013; Jakšić *et al.* 2015; Dragan *et al.*2019).

A study from Karachi, researched on 156 milk samples revealed 91.7% AFM1 contamination with a range of 20-3090ng/kg and a mean value of 346.2ng/kg. The study showed that 80.1% of samples exceeding the EU limit while almost 32.7% samples lying outside the limit of FDA limit (Asghar *et al.* 2018). Another study conducted in Southern Punjab, Pakistan explored seasonal effect of AFM1 in Milk samples. Analysis showed an overall contamination of 93% with a contamination range of 1-260ng/kg and 53% samples exceeding the EU limit (Ismail *et al.* 2016). The same study showed that maximum contamination was found in winter i.e., 92% contamination. Comparatively, our study was conducted in the months of summer, the season with high accessibility of green and fresh fodder. This is already proven by various studies that Milk samples from the months of summer showed less AFM1 contamination (Ghiasian *et al.* 2007; Peng and Chen, 2009). However, some contradictory evidence of an increase in the production of mycotoxins in elevated temperature conditions has been reported as well (Paterson and Lima, 2010). Similarly, year-long Serbian-based research claimed the highest AFM1 contamination in the season of autumn, reporting 29.3% of samples of raw milk exceeding the EU limit (Miocinovic *et al.* 2017).

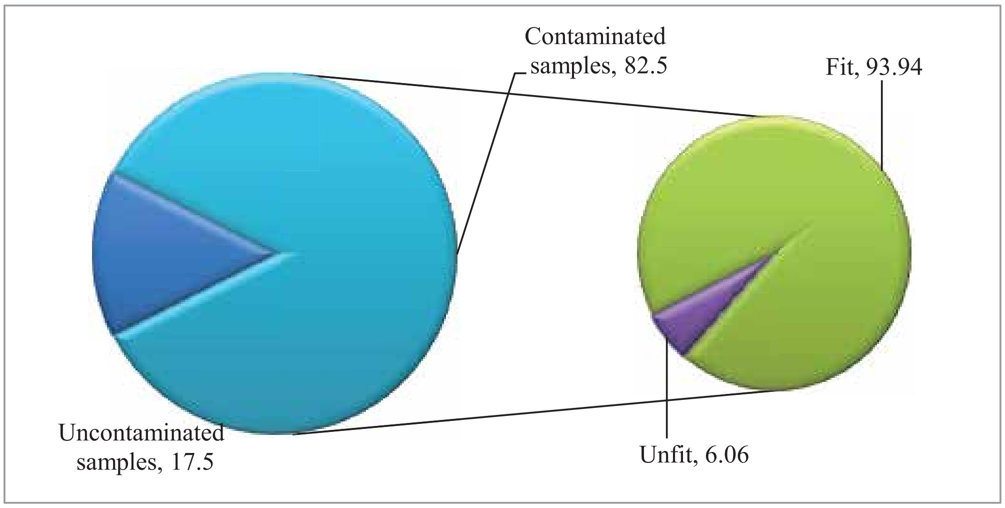

Fig. 2. FDA/PPFR samples analysis

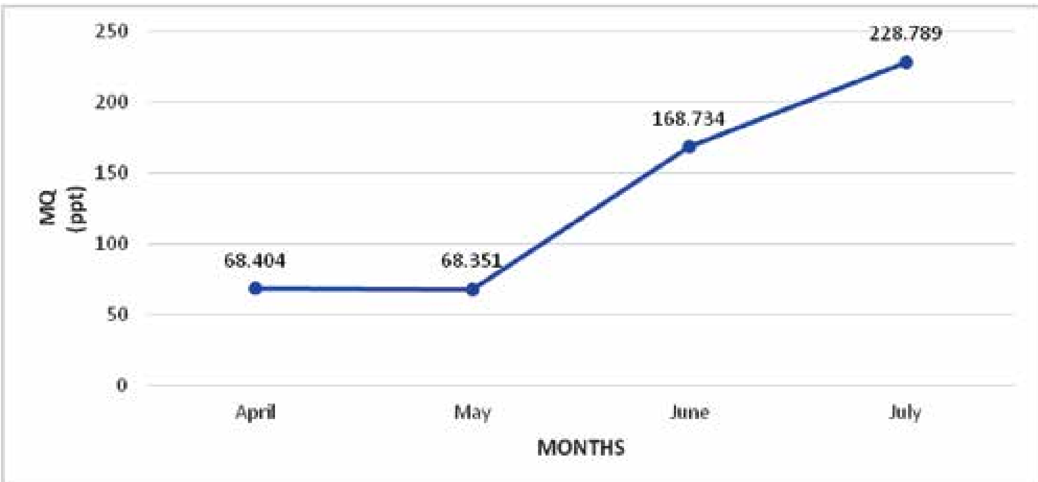

Fig. 3. Variation of AFM1 contamination throughout the Summer

Other than humidity and temperature, the feed fed to cattle also contributes greatly to the contamination of AFM1 in Milk. A study reported higher contamination of Aflatoxin B1 in such leftover breads and the feed being commercially given to cattle in Pakistan (Ismail *et al.* 2017). The same researcher previously reported that leftover bread is the primary source for direct intake of Aflatoxigenic fungal species in Pakistan (Ismail *et al.* 2016).

The presented results validate that record-breaking rainfall in Punjab contributed to more ideal growth of fungal species in fodder due to the humid environment, leading to more contamination of Aflatoxin M1 in Milk of Lahore.

Conclusion

The findings of the study conducted clearly shows that raw milk being sold at retail shops in Lahore is unfit for human consumption. Lahore being one of the largest cities with nearly 11,119,985 citizens (Pakistan Bureau of Statistics, 2017) poses a higher degree of contaminating its inhabitants with AFM1. The study showed that citizens of Lahore are at greater risk of being exposed to AFM1 in more humid months as compared to dry months. So, steps need to be taken from governance to the grass-roots level to ensure the quality of milk being sold especially in humid months.

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