Pakistan J. Zool., vol. 42(6), pp. 697-700, 2010.

Monitoring of Aflatoxin M_1 in Market Raw Milk in Lahore City, Pakistan

Khushi Muhammad*, Muhammad Yasin Tipu, Mateen Abbas, Abdul Muqeet Khan and Aftab Ahmad Anjum

Department of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan (KM, AAA), and Quality Operations Laboratory, UVAS, Lahore 54000, Pakistan (MYT, MA, AMK)

Abstract.- Aflatoxin M_1 (AFM_1) was determined in market raw milk samples collected from four towns of city Lahore, Pakistan. Total 84 samples were collected during a period of 4 months (April through July 2007) and were processed for purification of AFM_1 through an immunoaffinity column. Each sample was analyzed for AFM_1 using fluorescent detector of high performance liquid chromatography (HPLC). Eighty one percent milk samples contained AFM1 levels exceeding the American and European tolerance limits. The mean value of AFM_1 was 17.38µg/L ranged from 0.69 to 100.04 µg/L. High levels of AFM_1 in the raw milk samples is an enormous health risk factor for end consumers. There is need to improve storage conditions of feed ingredients that will mitigate the AFB_1 production in the feed/ration and ultimately decrease the AFM_1 levels in the animal milk.

Key Words: Aflatoxin M_1, HPLC, fluorescent detector, market raw milk samples.

INTRODUCTION

Milk and its products are fundamental components of human diet. Milk is mainly contaminated with aflatoxin M_1 (AFM_1). Consumption of such milk may be the principle way for entrance of AFM1 into the human body (Galvano *et al*., 2001). AFM1 is found in milk of the animals that are fed on aflatoxin contaminated feed (Van Egmond, 1989). Aflatoxin consists of B1, B2, G1 and G2 components and is produced as a result of growth of *Aspergillus flavus* and *Aspergillus parasiticus* species on feed ingredients during storage and/or transportation (Ali *et al*., 2005; Rizzi *et al*., 2003). Animals fed on aflatoxin contaminated feed metabolize in their liver AFB_1 into its hydroxylated product such as AFM_1 that is excreted in milk, feces and urine (Guerre *et al*., 2000). About 1-3% ingested AFB_1 is converted into AFM_1 (Ali *et al*., 1999; Barbieri *et al*., 1994).

The AFB_1 or AFM_1 toxins enter into food chain and accumulate in the adipose tissues of the end consumers. These toxins are considered the most serious threat to animal/human health due to their potential of carcinogenic, hepatotoxic, teratogenic and mutagenic (Maqbool *et al*., 2004; Wang *et al*., 1996; Wild and Turner, 2002).

The study was designed to monitor the AFM_1 in fresh raw milk retailed in Lahore city, Pakistan in terms of its compliance with international aflatoxin limits using an optimized HPLC method.

MATERIALS AND METHODS

Source of milk samples

Middle men collect fresh milk from dairy farmers from suburban and rural areas in morning and evening times and supply to retail milk shops of city Lahore, Pakistan. Eighty four samples of raw milk (250 ml each) were collected randomly from the markets of Gulberg Town, Ravi Town, Gungbuksh Town and Shalimar Town, during April 2007 to July, 2007. The samples (15 samples from each town) were collected once a year. The milk samples in sterilized polythene bags were transported in ice-packed cooler to the laboratory where were stored at -20C until analyzed for AFM_1.

Chemicals and reagents

Acetonitrile (HPLC grade) of Sigma-Aldrich (Steinheim, Germany) was used for AFM_1 analysis. The immunoaffinity columns AflaM_1 TM HPLC were obtained from VICAM (Watertown, MA, USA). The water used for analysis was double distilled with Millipore water purification system

* Corresponding author: drkhushimuhammad@hotmail.com
0030-9923/2010/0006-0697 $ 8.00/0

(Bedford, MA, USA). Standard of AFM1 (10 µg/mL in acetonitrile) was purchased from Supelco (Bellifonte, PA, USA). All the other chemicals used were of analytical grade.

Extraction procedure

The AFM_1 in milk was extracted using the method as described by Dragacci *et al*. (2001). Each of the stored milk samples was warmed in water bath at 37°C and centrifuged at 600x g for 10 minutes. The top layer with fat contents was removed and the milk was passed through filter paper (Whatmann No. 4). Fifty mL of the milk was loaded in a syringe barrel which was attached with immunoaffinity columns (IAC). The test portion was passed at the flow rate of 2-3 mL/min. The column was washed with 20 mL water and then air dried. Acetonitrile (4 mL) was passed through the column for at least 60 seconds and aflatoxin M_1 was eluted. The eluate was dried using water bath in fume hood. It was diluted with the mobile phase at the time of its determination through liquid chromatography (LC).

Determination of AFM_1 with fluorescence detection

Each sample was processed for determination of AFM_1 through HPLC system of Agilent 1100 series (Agilent, USA), using AOAC Official Method, 2000.08 (Dragacci *et al*., 2001). The HPLC system was equipped with an auto sampler LAS G1313A, and a fluorescence detector FLD G1321A with excitation and emission wavelength of 365 nm and 435 nm, respectively. The ZORBAX Eclipse XDB-C18 (Octadecyl silane chemically bonded to porous silica) column (Agilent, USA), 4.6 ×150 mm with particle size 5 µm in diameter and acetonitrile in ratio of 25% to 75% of water was used as mobile phase. The flow rate was set to be 0.8 mL/min. Standard solutions AFM_1 with concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/L in acetonitrile were used to obtain the calibration curve. The retention time for AFM_1 was 6.1 min.

Statistical analysis

The results regarding AFM_1 levels in milk samples were statistically analyzed by applying one way analysis of variance (ANOVA) (Steel and Torrie, 1997).

RESULTS AND DISCUSSION

Aflatoxin M_1 (AFM_1) was detected in raw market milk supplied to Lahore city, Pakistan. Presence of AFM_1 in milk and milk products is a worldwide concern since these products are main source for introducing aflatoxins in the human diet and are largely consumed by children including infants who are highly susceptible to adverse effects of the toxins (Rastogi *et al*., 2004).

Amount of AFM_1 contamination in raw milk samples was more than 50 ng/ kg (Table I). The levels ranged from 0.69 to100 µg/L in analyzed samples. Hussain and Anwar (2008) analysed milk samples from dairy animals at Punjab level and found 96.4 % samples having toxin level below the US tolerance limit. Only 3% samples were having the levels higher than the tolerance limits. Due to serious health concerns, many countries have set maximum permissible standards for levels of AFM_1 in milk and dairy products and AFB_1 in animal feeds. However, regulatory limits of permissible standards are influenced by economic considerations world wide (Stoloff *et al*., 1991; Van Egmond, 1989). The US regulation has prescribed a limit of 500 ng/kg of AFM_1 in milk and milk products (Codex Alimentarius Commission, 2001). However, European Communities has fixed the limit to a maximum of 50 ng/kg of AFM_1 (Commission Regulation EC No.466/2001). Similar high levels of the HPLC based AFM_1 levels were detected in Indonesia, Philippines and Thailand (Henry *et al*., 2001). In Iran, 98 samples were positive for AFM_1 with an overall mean level of 0.053 µg/L. Levels of the toxin were also higher in winter and spring than in summer and autumn (Tajkarimi *et al*., 2007) while in Sarab City of Iran, 77% (total 111 raw milk samples) were contaminated with AFM_1 levels (range between 0.015 and 0.280 µg/L) and 40% of the positive samples exceeded the tolerance limit of 0.050 µg/L (Kamkar, 2005). In Turkey, 47% of the 129 analyzed samples contained AFM_1 levels exceeding the EU accepted limit (Unusan, 2006). In North African countries, randomly selected samples of raw cow milk were contaminated with AFM_1 (rang between 30 and 3130 ng/L) (Elgerbi *et al*., 2004). In India, the incidence of contamination of AFM_1 in

Table I.- Incidence of Aflatoxin (M1) contaminated milk samples in different towns* of Lahore city, Pakistan.

Area (n=21)	Contaminated samples (%)	Minimum (µg/L)	Maximum (µg/L)	Mean (µg/L)	± SD	± SE
Gulberg Town	15 (71.4)	1.47	56.02	19.75[a]	18.29	4.89
Ravi Town	18 (85.7)	1.30	73.58	15.43[a]	16.78	4.07
Gungbuksh Town	17 (80.9)	0.98	40.46	12.13[a]	11.24	2.81
Shalimar Town	18 (85.7)	0.69	100.04	22.31[a]	26.18	6.35

* There are nine towns of Lahore city, Pakistan but milk samples were randomly collected from four towns of the city. Figures having similar superscript are not significantly different (P>0.05).

infant milk, milk based cereal weaning food and liquid milk samples was almost in the magnitude of 87% (Rastogi *et al.*, 2004), with 99% of contaminated samples exceeding the EU/Codex recommended limits.

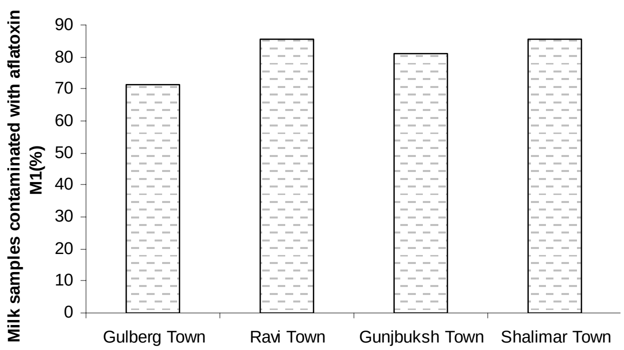

Fig. 1. Area-wise comparison of aflatoxin m1 in milk. A total of eighty four raw milk samples were collected from four towns of Lahore City, Pakistan (21 samples from each). Each positive sample means that it contains Aflatoxin M_1 (AFM_1) higher than the permissible limit (500 µg/L).

The incidence of AFM_1 contamination in the milk samples collected from Gulberg Town, Ravi Town, Gunjbukish Town and Shalimar Town was 71, 86, 81 and 86 percent, respectively. Milk samples from either of the town contained AFM_1 levels exceeding the permissible limits (Fig.1). Hussain *et al.* (2008) reported 42.5% and 52.5% milk samples were found contaminated from buffalo and cow, respectively, with the toxin. AFM_1levels in almost all the contaminated milk samples exceeded 50 ng/kg. These results are in accordance with El-Sayed *et al*. (2000), Salem (2002) and Elgerbi *et al*. (2004) who reported high levels of AFM_1 in bovine raw milk. Moreover, there is a strong correlation between AFM_1 level in milk and AFB_1 content in the animal's ration (Van Egmond, 1989). The most important factors affecting the amount of AFB_1 occurrence in feed was undoubtedly temperature and moisture. The toxin producing fungi such as *Aspergillus flavus* and *A. parasiticus* species show enormous growth in feeds having water contents between 13% and 18% and environmental moisture between 50% and 60%. Furthermore, these moulds can produce the toxin under conditions of 25°C and 85–90% relative humidity (Bakirci, 2001).The results indicate that feeds/ration for the dairy animals might be heavily contaminated with AFB_1.

Keeping in view high levels of AFM_1, there is dire need to improve storage conditions of animal ration/feed that will mitigate the AFB_1 levels in feed/feed ingredients and ultimately decrease the toxin in animal milk.

It is concluded that levels of AFM_1 in 81% raw milk samples collected from Lahore market exceeds the permissible limits. In Pakistan, more than 90 percent milk is consumed as raw so could be main source of the toxin for end users.

ACKNOWLEDGMENTS

The authors of the manuscript are highly thankful to Higher Education Commission, Islamabad, Pakistan, for funding the project in the best interest of dairy industry and end-consumers.

REFERENCES

ALI, N., HASHIM, N.H. AND YOSHIZAWA, T., 1999. Evaluation and application of a simple and rapid method for the analysis of aflatoxins in commercial foods from Malaysia and the Philippines. *Fd. Additi. Contam.*, **16**: 273–280.

ALI, N., HASHIM, N.H., SAAD, B., SAFAN, K., NAKAJIMA, M. AND YOSHIZAWA, T., 2005. Evaluation of a method to determine the natural occurrence of aflatoxins in commercial traditional herbal medicines from Malaysia and Indonesia. *Fd. Chem. Toxicol.*, **43**: 1763–1772.

BAKIRCI, I., 2001. A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. *Fd. Contr.*, **12**: 47–51.

BARBIERI, G., BERGAMINI, C., ORI, E. AND PESCA, P., 1994. Aflatoxin M1 in parmesan cheese: HPLC determination. *J. Fd. Sci.*, **59**: 1313–1331.

CODEX ALIMENTARIUS COMMISSION, 2001. *Comments submitted on the draft maximum level for Aflatoxin M1 in milk. Codex committee on food additives and contaminants* 33rd session, Hague, The Netherlands

COMMISSION REGULATION (EC) No. 466/2001, 2001. Setting maximum levels for certain contaminants in food stuffs. *Off. J. Europ. Commun.*, **L 077**: 1–13.

DRAGACCI, S., GROSSO, F. AND GILBERT, J., 2001. Immunoaffinity column cleanup with liquid chromatography for determination of aflatoxin M1 in liquid milk: collaborative study. *J. AOAC Int.*, **84**: 437–443.

ELGERBI, A.M., AIDOO, K.E., KANDLISH, A.A.G. AND TESTER, R.F., 2004. Occurrence of aflatoxin M1 in randomly selected North African milk and cheese samples. *Fd. Addit. Contam.*, **21**: 592–597.

EL-SAYED, A.A., NEAMAT-ALLAH, A.A. AND SOHER-EALY, 2000. Situation of mycotoxins in milk, dairy products and human milk in Egypt. *Mycotox. Res.*, **16**: 91–100.

GALVANO, F., GALOFARO, V., RITIENI, A., BOGNANNO, M., DE ANGELIS, A. AND GALVANO, G., 2001. Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy: second year of observation. *Fd. Addit. Contam.*, **18**: 644–646.

GUERRE, P., BAILLY, J.D., BERNARD, G. AND BURGAT, V., 2000. Excretion of lactee des mycotoxines: Quels risques pour Ie consommatuer? *Rev. Med. Vet.*, **151**: 7-22.

HENRY, S.H., WHITAKER, T.B., RABBANI, I., BOWERS, J., PARK, D., PRICE, W., BOSH, F.X., PENNINGTON, J., VERGER, P., YOSHIZAWA, T., VAN EGMOND, H., JONKER, M.A. AND COKER, R., 2001. *Aflatoxin M1, Chemical Safety Information from Inter Government Organizations,* <www.inchem.org/ documents/ jecfa/ jecmono /v47je02.htm>.

HUSSAIN, I. AND ANWAR, J., 2008. A study on contamination aflatoxin M1 in raw milk in the Punjab Province of Pakistan. *Fd. Contr.*, **19**: 393-395.

HUSSAIN, I. ANWAR, J., MUNAWAR, A.M. AND ASI, M.R., 2008. Variation of levels of aflatoxin M1 in raw milk from different localities in in the central areas of Punjab, Pakistan. *Fd. Contr.*, **19**: 1126-1129.

KAMKAR, A., 2005. A study on the occurance of aflatoxin M1 in raw milk produced in Sarab city of Iran. *Fd. Contr.*, **16**: 593–599.

MAQBOOL, U., AHMAD, M., ANWAR-UL_HAQ, H. AND IQBAL, M.M., 2004. Determination of aflatoxin-B1 in poultry feed and its components employing enzyme linked immunosorbent assay (ELISA). *Toxicol. environ. Chem.*, **86**: 213–218.

RASTOGI, S., DWIVEDI, P.D., KHANNA, S.K. AND DAS, M., 2004. Detection of aflatoxin M1 contamination in milk and infant milk products from Indian markets by ELISA. *Fd. Contr.*, **15**: 287–290.

RIZZI, L., SIMIOLI, M., RONCADA, P. AND ZAGHINI, A., 2003. Aflatoxin B1 and clinoptilolite in feed for laying hens. Effect on egg quality, mycotoxin residues in livers and hepatic mixed function oxidase activities. *J. Fd. Protect.*, **66**: 860–865.

SALEM, D.A., 2002. Natural occurrence of aflatoxins in feedstuffs and milk of dairy farms in Assuit Province, Egypt. *Wiener-Tieraerztl. Monatsschr.*, **89**: 86–91.

STEEL, R.G.D. AND TORRIE, J.H., 1977. *Principles and procedures of statistics.* 2nd ed. McGraw Hill, New York. pp. 137-167.

STOLOFF, L., VAN EGMOND, H.P. AND PARK, D.L., 1991. Rationales for establishment of limits and regulations for mycotoxins. *Fd. Addit. Contam.*, **8**: 213–222.

TAJKARIMI, M., ALIABADI-SH, F., SALAH-NEJAD, M., POURSOLTANI, H., MOTALLEBI, A.A. AND MAHDAVI, H., 2007. Seasonal study of aflatoxin M1 contamination in milk in five regions in Iran. *Int. J. Fd. Microbiol.*, **116**: 346–349.

UNUSAN, N., 2006. Occurrence of aflatoxin M1 in UHT milk in Turkey. *Fd. Chem. Toxicol.*, **44**: 1897–1900.

VAN EGMOND, H.P., 1989. Current situation on regulations for mycotoxins. Overview of tolerances and status of standard method of sampling and analysis. *Fd. Addit. Contam.*, **6**: 139-188.

WANG, L.Y., HATCH, C.J., CHEN, C.J., LEVIN, B., YOU, S.L. AND LU, S. N., 1996. Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. *Int. J. Cancer*, **67**: 620-625.

WILD, C.P. AND TURNER, P.C., 2002. The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis*, **17**: 471-481.

(Received 30 January 2010, revised 12 April 2010)