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Seasonal prevalence level of aflatoxin M_1 and its estimated daily intake in Pakistan

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ABSTRACT

Aflatoxin M_1 level was measured in 520 milk samples during autumn, winter, spring and summer seasons of 2013–14 in five districts of Southern Punjab-Pakistan. Analyses were performed by using enzyme linked immuno sorbent assay (ELISA) method. Aflatoxin M_1 was found positive in 93% milk samples in the range of 0.001–0.26 μg/l while 53% samples were found to exceed the European Union maximum Limit for aflatoxins M_1 i.e. 0.05 μg/l. The seasonal prevalence level of AFM_1 was found in the order of winter > spring > autumn > summer. Comparing the aflatoxin M_1 level during different day times, morning milk was found 37–50% more contaminated than evening milk. The estimated daily intake (EDI) of aflatoxins M_1 during different seasons of year for various age groups was found in the range of 0.22–5.45 ng/kg/day. Infants were found in the highest risk group while adults were in the lowest. The results of the study indicate that people of Pakistan are at high risk of health issues related with aflatoxins M_1.

1. Introduction

Aflatoxins are the secondary metabolites of four different species of *Aspergillus* including *A. flavus*, *A. nomius*, *A. parasiticus* and *A. fumigatus*. There are 18 different types of aflatoxins among which the most prominent in the order of toxicity are aflatoxins B_1, aflatoxins B_2, aflatoxins G_1 and aflatoxins G_2, respectively. The toxic effects of aflatoxins on humans include carcinogenicity, teratogenicity, genotoxicity and cytotoxicity (IARC, 2002; Neal, Eaton, Judah, & Verna, 1998; Sassahara, Pontes, & Yanak, 2005; Shibahara, Ogawa, Ryo, & Fujikawa, 1995). Aflatoxins are reported in a number of food commodities including cereals, fruits, nuts, cottonseeds and milk. Due to the favorable environmental conditions and poor implementation of regulations the aflatoxins above the permissible limits are mostly reported in developing nations like Pakistan, Iran and India (Asi, Iqbal, Ariño, & Hussain, 2012; Darsanaki, Aliabadi, & Chakoosari, 2013; Siddappa, Nanjegowda, & Viswanath, 2012).

The ingestion of aflatoxins B_1 and B_2 contaminated fodder by the dairy animals results in aflatoxin M_1 (AFM_1) and M_2 (AFM_2), respectively, in the milk of animal due to a conversion process by the liver of animal. In 2002, the international agency for research on cancer (IARC, 2002) classified AFM_1 as a group 1 human carcinogen despite of being ten times less toxic than AFB_1. The highly toxic nature of AFM_1 is due to its presence inside milk which is more frequently consumed by the most vulnerable group of people i.e. children and elderly (Paniel, Radoi, & Marty, 2010). The conversion of AFB_1 into AFM_1 depends on a number of factors including the season of the year, breed of the animal, milking time and the history of infection and feeding (Anfossi, Baggiani, Giovannoli, & Giraudi, 2011). The conversion rate of AFB_1 into AFM_1 ranges between 0.3 and 6% (Var & Kabak, 2009) while these toxins in the milk of animal can be detected after 12–24 h of contaminated feeding (Prandini et al., 2009).

Milk due to the presence of all basic nutrients is the basic food item for mankind starting right from the birth and goes till the end of their lives (Galvano, Galofaro, & Galvano, 1996). Due to its consumption by the most vulnerable age group serious regulatory limits are established globally for AFM_1 in milk ranging between 0.05 and 0.5 ppb. The most commonly adopted permissible limit for AFM_1 in milk is 0.05 ppb set by Codex Alimentarius Commissions

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(2001) while the US limit for AFM_1 is 0.5 ppb.

Present study focuses on the detection of AFM_1 in bovine milk during three different periods of the year. Level of AFM_1 was measured in morning and evening milk samples. Moreover, the estimated daily intake level of AFM_1 through milk during different seasons of year by different age groups was also calculated.

2. Materials and methods

2.1. Sample collection

A total of 520 milk samples were collected from the dairy farms situated in five districts of Southern Punjab including Multan, Bahawalnagar, Rahim Yar Khan, Dera Ghaazi Khan and Sahiwal during September 2013 to August 2014. Milk samples were collected at two different day times to compare the AFM_1 level during morning (262) and evening milk samples (258). Milk samples (1 L) were collected in clean glass bottles and were transported to the lab, in an ice box. Samples were analyzed immediately or kept in freezer until analysis.

2.2. Aflatoxin M_1 kit & sample preparation

The enzyme linked immuno sorbent assay (ELISA) kit for AFM_1 was supplied by Euro Proxima, Netherlands (catalogue No. 5121AFM). The kit contained antibody coated microtiter plates, buffer solutions, substrate solution, stop solution, conjugate solution, aflatoxins M_1 free skim milk, and standard solutions: 0, 0.006, 0.012, 0.025, 0.05, 0.1 and 0.2 μg/l. Milk samples were prepared according to the guidelines of ELISA kit manual. Briefly, the milk samples were defatted by centrifugation for 10 min at 2000 × *g* by using centrifuge machine of Hedolf (Germany). The upper fat layer was removed through a glass Pasteur pipette. From the defatted portion, 100 μl of the sample was used for ELISA assay. All the standards, samples and blank were pipette in duplicate. The ELISA reader used for measuring the absorbance was Bio-Tek ELx800 (Indonesia).

2.3. Assay protocol

Quantitative analysis for AFM_1 in milk was performed by following the guidelines provided in the ELISA kit manual. The standard solutions, blank and defatted milk samples were added (100 μl each) in the wells of microtiter plate. The plate was sealed and shaken for a few seconds on a microtiter plate shaker. The plates were incubated in dark for an hour at room temperature. After the given time, the remaining solution was discarded and the plates were washed 3 times with rinsing buffer. Now, 100 μl of conjugate solution was added in each well, except wells of blank. The plate was again incubated in dark at room temperature for 30 min. The remaining solution was discarded and the plates were washed 3 times with rinsing buffer. Substrate solution (100 μl) was pipette into each well and the plates were incubated for 30 min at room temperature. After incubation, 100 μl of stop solution was added to each well. The absorbance was measured through ELISA reader at 450 nm.

2.4. Evaluation of data

The limit of AFM_1 detection (LOD) for ELISA assay was 0.001 ng/ml. The mean absorbance values of samples and standard solutions were divided by the mean absorbance value of zero standard (maximal absorbance) and multiplied by 100.

$$\%\ \text{maximal absorbance} = \frac{\text{Mean absorbance value of standard or sample}}{\text{Mean absorbance value of zero standard}} \times 100$$

The concentration of AFM_1 for each sample was calculated by using the standard calibration curve. For the statistical evaluation of the data Statistix 8.1 (Statistix Inc., Florida, USA) software was used. In order to compare the data of different treatments one way analysis of variance (ANOVA) was applied. A probability level of $P < 0.05$ was adopted for the consideration of differences as statistically significant. Microsoft Excel (2010 version) was used for the computation of mean values and standard deviations (SD).

2.5. Verification method

A standard solution of AFM_1 was purchased from Sigma chemicals (A6428) for validation purpose. The standard solution was added in AFM_1 free milk samples at the concentrations of 0.01, 0.02, 0.05 and 0.1 μg/l. The recovery percentages for AFM_1 ranged between 96.1 and 98.7% while the variation coefficient was found in the range of 2.1–4.3% (Table 1).

2.6. Estimated daily intake of AFM_1

The estimated daily intake (EDI) of AFM1 through milk consumption was calculated on the basis method proposed by Cano-Sancho, Marin, Ramos, Peris-Vicente, and Sanchis (2010). The daily intake level of milk by five different age groups of male and female was estimated based on a food frequency questionnaire involving 495 individuals. Following formula was adopted for calculating mean μg/kg/day intake of AFM_1

$$\text{EDI}\ (\mu g/kg/day) = \frac{\text{Daily intake level of milk}\ (kg/day) \times \text{Aflatoxin M}_1\ \text{level in milk}\ (\mu g/L)}{\text{Average individual weight}\ (kg)}$$

3. Results and discussion

3.1. Variation in aflatoxin M_1 level during different seasons of year

AFM_1 level was measured in bovine milk samples during all seasons of a year including autumn, winter, spring and summer