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Assessment of aflatoxin M_1 in milk and milk products from Punjab, Pakistan

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ABSTRACT

The basic objective of this research was to investigate the incidence and occurrence of aflatoxin M_1 (AFM_1) in milk and dairy products produced in the province of Punjab, Pakistan. Milk samples (107) and dairy products including yogurt (96), white cheese (119), cheese cream (150) and butter (74) samples were analyzed using High performance liquid chromatography equipped with fluorescence detector. The results have shown that AFM_1 was detected in 71% of milk samples with 58% samples were found above the permissible limit of European Union (EU). AFM_1 was detected in 61% of yogurt, 78% white cheese, 59% cheese cream and 45% butter samples with 47, 15, 11 and 52% samples of yogurt, white cheese, cheese cream and butter, respectively were found above the recommended limit of EU. The data of the present study will be helpful for the implementation of regulatory limit for AFB_1 in order to minimize or avoid AFM_1 in milk and milk products from Pakistan and also gives insights that whether the occurrence of AFM_1 in dairy products was considered as a possible risk for consumers health.

1. Introduction

In milk producing countries of the world, Pakistan stands fourth with production of 45 billion liters of milk per annum. More than 55 million people in Pakistan are directly dependent on livestock for their livelihood (Iqbal, Asi, & Ariño, 2011). Buffalo is the major milk-producing animal in Pakistan, and this country stands second in world's buffalo milk production (Hussain, Ghafoor, & Saboor, 2010). The dairy industry of Pakistan is facing a number of problems such as lack of dairy-related education and lack of financial and infra-structure facilities, but the quality and contamination of toxins like mycotoxins (especially AFM_1) check is the most neglected aspect of the whole system (Jalil, Rehman, Sial, & Hussain, 2009).

Mycotoxins are a group of naturally occurring secondary metabolites which are mainly produced by the filamentous fungi (Iqbal et al., 2011). Among mycotoxins, aflatoxins (AFs) are the most toxic and carcinogenic class and are mainly produced by fungi *Aspergillus flavus*, *Aspergillus parasiticus*, and rarely by *Aspergillus nomius*. They can contaminate food, vegetable, fruits, cereals and cattle feed (Asi, Iqbal, Ariño, & Hussain, 2012). AFs are associated with the incidence of certain types of cancer which poses a global concern over food and feed safety (Gong et al., 2002, 2004; Turner, Moore, Hall, Prentice, & Wild, 2003).

AFM_1 is recognized as a metabolite of AFB_1 and is secreted in milk of mammals that ingested contaminated feed (Ruangwises & Ruangwises, 2010). Due to high toxicity of AFM_1, it was initially classified by the International Agency for Research on Cancer (IARC) as an agent in Group 2B, with a possible carcinogenic effect on humans (IARC, 1993). However, it was reclassified as Group 1 carcinogenic agent (IARC, 2002), although its carcinogenicity is approximately 2–10% of AFB_1 (Asi et al., 2012; Creppy, 2002).

Milk and dairy products provide important nutrients for humans; especially children are regularly included them in their diet (Baskaya, Aydin, Yildiz, & Bostan, 2006). Kuiper-Goodman (1990) has reported a tolerable daily intake of 0.2 ng/kg b.w. for AFM_1. In order to minimize the risk of aflatoxicosis, strict regulatory limits are currently implemented in most of the countries. In the European Commission Directive (2004), the maximum AFM_1 content in liquid milk and dried or processed milk products intended for adults has been set 50 ng/l and 25 ng/l for milk intended for infants (European Commission Regulation, 2004). The US Food and Drug Administration however, declared the maximum permissible level of 0.5 g/kg in milk (Kav, Col, & Tekinşen, 2011). The Codex Alimentarius legal limit for AFM_1 in butter and cheese are 50 ng/kg and 250 ng/kg, respectively (Codex Alimentarius Commission, 2001). There are thus differences in the maximum tolerance limit for AFM_1 in various countries, and many including Pakistan have no legal limit for AFM_1 in milk or dairy products (Iqbal et al., 2011).

Several studies have been undertaken to investigate the occurrence of AFM_1 in milk (Hussain, Anwar, Asi, Munawar, & Kashif, 2010; Hussain, Anwar, Munawar, & Asi, 2008; Iqbal et al., 2011) and to analyze the seasonal variation of AFM_1 in milk (Asi et al.,

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2012; Hussain & Anwar, 2008) from Pakistan. However, very limited data is available on the incidence of AFM_1 in milk products like yogurt, white cheese, cheese cream and butter from Pakistan (Maqbool, Anwar-Ul-Haq, & Ahmad, 2009). The objectives of the present study were to provide the incidence level of AFM_1 in milk as well as in milk products and to correlate the contamination level with EU recommended limits. The conclusion and recommendations drawn from this study will be helpful for policy makers in central and local governments, extension agents to implement the strict regulation on AFB_1 in food and feed to reduce or avoid the contamination of AFM_1 in milk and dairy products.

2. Materials and methods

2.1. Sampling

A total of 546 samples of raw milk and milk products were randomly collected during November 2010 to April 2011 from main districts of Punjab, Pakistan (as shown in Fig. 1). The samples of milk and milk products were collected from milking sites, small and large dairies and dairy farmhouses. The sampling sites visited were randomly selected from those situated in the most populated areas in the districts to the least populated. Total 107 samples of milk, 96 yogurt, 119 white cheese, 150 cheese cream, and 74 butter were collected for the analysis of AFM_1. The share of Punjab, province in total milk production is 62%. The size of milk samples were at least 1 L while yogurt, white cheese, cheese cream and butter samples were at least of 500 g. The samples were collected in plastic bags and during collection and transportation, the milk and dairy samples were kept in an icebox. The samples were either analyzed immediately or stored in a freezer at −4 °C in case of delayed analysis.

2.2. Chemicals and regents

Standard of AFM_1 (10 μg/l in acetonitrile) and acetonitrile (HPLC grade) were purchased from Sigma–Aldrich, Steinheim, Germany while, immunoaffinity columns (IAC) $AflaM_1$™ from VICAM, Watertown, MA, USA. A standard curve for AFM_1 was prepared by diluting the standard with acetonitrile into different concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/l solutions and stored in caped vials in a refrigerator at −4 °C until further analysis. In present study double-distilled water was used and all other chemicals and reagents were at least of analytical grade.

2.3. Extraction

2.3.1. Extraction of milk samples

The extraction of AFM_1 from milk samples were carried out by our previously validated method (Asi et al., 2012), with some modifications. The temperature of liquid milk samples were maintained at 37 °C by placing them in a water bath set at 37 °C and then samples were centrifuged at 2500 rpm for 5 min to separate the fat layer. After centrifugation, samples were filtered through Whatman No. 5 filter paper and 50 ml of filtrate was transferred into a syringe barrel attached to an IAC and passed at a rate of 2 ml/min using solid phase extraction manifold. The column was washed with 20 ml double distilled water to eliminate impurities and then, AFM_1 was eluted with 4 ml of pure acetonitrile at a rate that it can be in contact with the column approximately 60 s. Finally, the eluate was evaporated to dryness using a gentle stream of nitrogen at 40 °C and then, the residues were diluted with 1 ml of mobile phase at the time of HPLC analysis.

2.3.2. Extraction of white cheese, cheese cream, yogurt and butter samples

The analysis of AFM_1 in yogurt, cheese and butter were reported first time from Pakistan using HPLC therefore, the method was validated as reported by (Kamkar, Karim, Aliabadi, & Khaksar, 2008). Briefly, 10 g sample (white cheese, cheese cream, butter or yogurt) and 10 g Celite (Sigma–Aldrich) in 80 ml of dichloromethane were blended for 3 min and then centrifuged at 21,000 rpm for 4 min to form slurry. After centrifugation, the slurry was filtered with Whatman no. 5 filter paper and the filtrate was evaporated to dryness under nitrogen stream at 40 °C. After evaporation, the residues were dissolved in 10 ml mixture of methanol, water and n-hexane (3:5:2 v/v/v). The aqueous phase was separated using a separator funnel and 5 ml of the aqueous filtrate from each sample was passed through an immunoaffinity column. Other components of the sample matrix were washed twice with 10 ml of water. The toxin was slowly removed from the column, twice with 2.5 ml of acetonitrile. Finally, the extracts were evaporated to dryness again under nitrogen stream at 40 °C and then, the residues were re-dissolved in 1 ml of mobile phase. The volume of injection into HPLC was 20 μl.

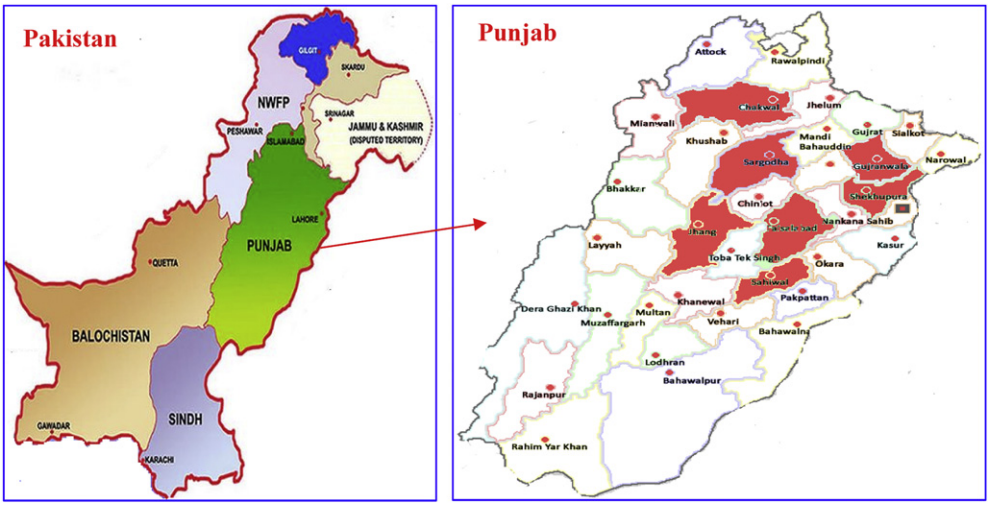

Fig. 1. Geographical locations of sampling sites of milk and milk products from Punjab, Pakistan.