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Original Article

A survey of aflatoxin M1 in cow milk in Southern Iran

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ABSTRACT

The competitive enzyme-linked immunosorbent assay technique was used to evaluate aflatoxin M1 (AFM1) levels in 168 samples of raw milk (135 samples and 33 samples from bulk tanks of farms and milk collection centers, respectively) and 12 samples of pasteurized milk in Fars province, Southern Iran. AFM1 was found in 55.56% of the samples with a mean concentration of 21.31 ng/L. The concentration of AFM1 in raw milk samples from farms was significantly ($p < 0.05$) lower than that in samples from collection centers and pasteurized milk. The concentration of AFM1 was not influenced by season, location, or type of farm. The concentrations of AFM1 in all samples were lower than the Iranian national standard limit (100 ng/L), but in 30% of raw cow milk samples they were higher than the maximum tolerance limit accepted by the European Union (50 ng/L); therefore, more effort is needed to control AFM1 levels in milk produced in Southern Iran.

1. Introduction

The presence of aflatoxins in food and feed is of great concern worldwide because of the health issues they can cause [1]. Aflatoxins are produced mainly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, and rarely by *A. nominus*, *A. tamarii*, or *A. pseudotamarii* strains when temperatures are between 24°C and 35°C and moisture content exceeds 7% [2–4]. Among the aflatoxins (B1, B2, G1, and G2), aflatoxin B1 (AFB1) is the most prevalent and potent natural carcinogen [5]. The presence of AFB1 in feeds and the subsequent access of lactating animals to it lead these animals to metabolize it to 4-hydroxylated form in their liver and excrete it as aflatoxin M1 (AFM1) in milk, urine, and feces [6,7]. About 0.3–6.2% of AFB1 in animal feeds is converted to AFM1, and it can be found in milk 12 hours after first ingestion and decreases to an undetectable level 72 hours after last ingestion of AFB1 [8,9].

Although previously AFM1 was assigned to group 2B (agents that are possibly human carcinogens) by the International Agency for Research on Cancer [10], it was thereafter reassigned to group 1 (class of agents that are certainly human carcinogens) for demonstrated toxic and carcinogenic effects [11]. A review of the literature shows that aflatoxins are most commonly known for causing acute or chronic liver disease depending on the doses used, but they are also considered immunosuppressive, hepatotoxic, mutagenic, teratogenic, and carcinogenic [2,12].

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Milk is one of the main foodstuffs in human diet especially for infants and children. Most studies indicate that processes such as pasteurization, sterilization, evaporation, concentration, or drying do not cause an appreciable change in the concentration of AFM1 in the product [7]. The AFM1 level in milk may vary according to geographic location, development level of the country, and climatic conditions; thereupon, it is important to determine its levels in produced milk in different locations to protect consumers from its harmful effects [13]. The maximum limits for AFM1 in raw milk vary in different countries depending on risk assessment and economic considerations. In the European Union (EU), the maximum level of AFM1 in liquid milk has been prescribed as 50 ng/L, whereas for United States and most of Asian countries' regulations it is 500 ng/L, which is higher than the maximum permissible level of 100 ng/L set by the Institute of Standards and Industrial Research of Iran [14–17].

Enzyme-linked immunosorbent assay (ELISA) is the quickest and simplest method for monitoring AFM1 in milk with good sensitivity, high precision, and optimal recovery [18].

The presence of AFM1 in milk has been shown in several surveys conducted in different regions of Iran using thin layer chromatography [19,20], high-performance liquid chromatography [21–23], or ELISA [24–34], and also in different countries worldwide: Brazil [13], Portugal [35], Spain [36], Lebanon [37], Syria [38], Turkey [39–42], Pakistan [43–45], South Korea [46], Sudan [47], Egypt [48], Morocco [49,50], Thailand [51], Indonesia [52], India [53], China [54], Serbia [1,55], and Croatia [56,57]. However, no published research is available on AFM1 levels in produced raw milk in Fars province. Annually, 497,000,000 L of milk is produced in Fars province, which ranks fifth in the country and first in the southern provinces of Iran [58]. The objective of this study was to determine the level of AFM1 in produced raw milk and to investigate its geographical and seasonal difference in Fars province (south of Iran).

2. Methods

2.1. Study area

A total of 192 milk samples were collected from three different areas in Fars province and labeled Sh, M, and S for Shiraz, Marvdasht, and Sepidan districts, respectively. Raw milk of cows from smallholder farms has was collected by milk collection centers, whereas it was transported to dairy factories directly by industrial dairy farms in Fars province. In each of these areas, raw milk was sampled from the bulk tank of three industrial dairy farms, three milk collection centers, and nine smallholder dairy farms (3 smallholder dairy farms that sold their milk to selected milk collection centers) seasonally. In each season, three pasteurized milk samples produced by dairy factories in Fars province were taken.

2.2. Milk sample preparation

Fresh milk samples (500 mL) were taken directly from storage tanks of farms or milk collection centers and pasteurized milk samples were bought from supermarkets. These samples were transported to the laboratory in ice boxes and stored in the dark at −18°C until the time of analysis. Milk samples were chilled at 10°C, of which 2 mL was centrifuged for analysis at 3500 rpm for 10 minutes at 4°C. As aflatoxins are water-soluble compounds [59], the upper creamy layers were completely discarded, and the lower phases were used for the quantitative test.

2.3. AFM1 measurement

The quantitative analysis of AFM1 was performed by competitive ELISA using an AFM1 kit (RIDASCREEN; R-Biopharm AG, Darmstadt, Germany). It had the following characteristics: detection limit, 5 ng/L; recovery rate, 95%; cross-reactivity, AFM1 100% and AFM2 30%; standard solutions, 0, 5, 10, 20, 40, 80 ng/L. The basis of the test was the antigen–antibody reaction. The wells in the microtiter strips were coated specific to AFM1 and filled with 100 μL of prepared samples or standard solutions. Antibodies were proportionally bound by shaking the plate gently and incubating at room temperature for 30 minutes in the dark. The wells were filled with 250 μL washing buffer after the complete removal of liquids. Then washing buffer was poured out, and this washing step was repeated twice. In the next step, 100 μL peroxidase conjugated AFM1 was added to the wells. Free antibodies were bound by conjugated AFM1 and any unbound enzyme conjugated AFM1 was removed by a washing step. Then, 100 μL of substrate and chromogen was added to wells and mixed gently by shaking the plate manually and incubated at room temperature for 15 minutes in the dark. Colorless chromogen was converted to blue by bound enzyme conjugate. Finally, 100 μL of 1N H_2SO_4 was added to wells, which led to a color change (from blue to yellow) [37]. The absorbance was measured at 450 nm in an ELISA plate reader (BioTek, Winooski, VT, USA). The absorption intensity was inversely proportional to the AFM1 concentration in the sample. A special software (RIDA SOFT Win; R-Biopharm AG) was used to draw standard curve and evaluate assays. The considered limit for positive samples was 5 ng/L AFM1.

2.4. Statistical analysis

All statistical analyses were carried out in SPSS for Windows 16.0.0 (SPSS Inc., 2007, Chicago, USA). Data were analyzed descriptively in the first step. Univariate analysis of variance was applied with AFM1 values as dependent variable and season, city, and herd type as independent variables. The means of AFM1 values was compared by using Duncan test. The relationship between contamination percentage and season or location in each type of farms was investigated using the chi-square test.

3. Results

Twelve raw milk samples were missed, and only 168 samples of raw milk (135 and 33 from bulk tank of farms and milk collection centers, respectively) and 12 samples of pasteurized milk were analyzed for AFM1. An exponential correlation was obtained by plotting the percentage of absorbance (y) and concentration (x) of AFM1 ($y = 96.72 - 10.2x$, with $R^2 = 0.991$) on