Assessment of aflatoxin M1 contamination in the milk of four dairy species in Croatia

Nina Bilandžić a,*, Durdica Božić a, Maja Đokić a, Marija Sedak a, Božica Solomun Kolanović a, Ivana Varenin a, Zeljko Cvetic b

a Laboratory for Residue Control, Department of Veterinary Public Health, Croatian Veterinary Institute, Savska cesta 143, HR-10000 Zagreb, Croatia
b General Department, Croatian Veterinary Institute, Savska cesta 143, HR-10000 Zagreb, Croatia

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A B S T R A C T

Aflatoxin M1 (AFM1) concentrations were measured in bulk cow milk samples from eastern Croatia, and in cow, goat, sheep and donkey bulk milk samples from other parts of Croatia during the period July–September 2013. AFM1 levels in milk were measured in the ranges (ng/L): cow 3.65–162.3 (eastern Croatia) and 2.69–44.9 (other regions of Croatia); goat 2.78–40.8; sheep 2.11–5.87; donkey 4.33–10.4. The concentration of AFM1 exceeded the EU MRL in 6.7% of cow milk samples from eastern Croatia. The highest level measured was 162.3 ng/L. AFM1 levels exceeded the LOQ value (23.2 ng/L) in only 59 samples of cow milk and two samples of goat milk of the total 402 samples analysed. A significant difference was found between the mean AFM1 concentrations of cow milk from eastern and other regions of Croatia (P < 0.05). The elevated AFM1 levels in cow milk from eastern Croatia indicate the use of contaminated supplementary feedstuff in some farms during the study period.

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1. Introduction

Mycotoxin aflatoxins produced by mould species of the genus Aspergillus are among the most investigated groups of contaminants in different types of food and feed. Approximately 18 aflatoxins have been identified. Among them, aflatoxin B1 (AFB1) is a highly toxic, mutagenic, teratogenic and carcinogenic compound that causes DNA damage, gene mutation, chromosomal anomalies and cell transformation, and therefore has been classified as a class I human carcinogen (carcinogenic) (IARC, 1993, 2002). The monohydroxylated derivate of AFB1, aflatoxin M1 (AFM1), is formed in the liver and excreted into the milk of lactating animals. Humans can be exposed following the ingestion of AFB1 contaminated food (Fallah, Jafari, Fallah, & Rahnama, 2009; Murphy, Hendrich, Landgren, & Bryant, 2006). In comparison with AFB1, the carcinogenicity of AFM1 is about ten times less (JECFA, 2001). Until 2002, AFM1 was classified as a class 2B substance (possible carcinogen) (IARC, 1993). However, following numerous studies concerning the carcinogenic, teratogenic, genotoxic and immunosuppressive influence on humans, AFM1, together with the aflatoxins B2, G1 and G2, was reclassified as a class I compound (IARC, 2002).

Climatic conditions regarding temperature and moisture in tropical and subtropical regions favour the growth of the toxigenic Aspergillus (Asi, Iqbal, Ariño, & Hussain, 2012; Elzupir & Elhussein, 2010; Picinin et al., 2013). Also, long periods of high temperatures and long-lasting drought in summer in other climatic regions, e.g. the northern temperature zone, may also favour the development of mycotoxins (Bilandžić et al., 2014; Decastelli et al., 2007). Respectively, variations of AFM1 levels have been determined during different seasons, with higher concentrations in winter than in summer (Iqbal, Jinap, & Asi, 2013; Nemati, Mehran, Hamed, & Masoud, 2010; Tajkarimi et al., 2008). This difference is influenced by a shortage of fresh green feed and the use of concentrated feed (contaminated with high levels of AFB1) with greater amounts of mixed supplementary feedstuff, dry hay and corn in dry periods or during winter months. As a result of contamination, AFM1 is excreted in the milk of contaminated lactants (Fink-Gremmels, 2008).

Milk is one of the main components in the human diet and is essential for infants and small children. Because AFM1 is not destroyed in production processes or in pasteurization or ultra-high temperature treatments, it is of great importance to provide the most effective control of raw milk and dairy products in accordance with the defined maximum residue levels set by the EU. The maximum residue for AFM1 concentrations in milk, heat-treated milk and milk for dairy products approved by the European...
Union (EU MRL) is 50 ng/kg, and 25 ng/kg for milk-based baby food (European Commission, 2006). However, higher MRL levels are permitted in other countries, e.g. 200 ng/L in Syria (FAO, 2004) and 500 ng/L in the USA (FDA, 2005), Brazil (BRASIL, 2002), China (Moh, 2011) and Serbia (Anonymous, 2013).

Numerous studies on AFM1 levels in raw cow milk have been conducted in recent years (Duarte et al., 2013; Piccin et al., 2013; Siddappa, Nanjegowda, & Viswanath, 2012; Xiong, Wang, Ma, & Liu, 2013). Also, several comparative studies of AFM1 levels in cow, goat and sheep milk have been conducted in Iran, Pakistan and Lebanon (Asi et al., 2012; Fallah, Rahnama, Jafari, & Saei-Dekordi, 2011; Hassan & Kassaify, 2014). In Croatia, several studies have been conducted on AFM1 concentrations in cow milk (Bilandžić, Varenina, & Solomun, 2010; Bilandžić et al., 2014; Markov, Frece, Cvek, Lovrić, & Delas, 2010) and one in sheep milk (Duraković, Mrkonjić-Fuka, Skelin, Duraković, & Redžepović, 2013).

Due to the identification of elevated concentrations of AFM1 in cow milk from eastern Croatia in the winter—spring season of 2013, this study compared AFM1 concentrations in milk from farms in this area and other regions of Croatia in the period July—September 2013. The second aim of the study was to detect the occurrence of AFM1 in raw milk of other lactating species, i.e. sheep, goat and donkey, collected from small family farms during the same period.

2. Materials and methods

2.1. Sample collection and preparation

A total of 337 bulk samples of cow milk were collected from small farms in eastern Croatia (n = 194) and other regions of Croatia (n = 143) during July—September 2013. Also, bulk samples of raw goat (n = 32), sheep (n = 19) and donkey (n = 14) milk were obtained from small farms from different regions of Croatia in the same period.

After collection at farms, milk samples were kept at 2–8 °C. Upon arrival at the laboratory, samples were frozen at −20 °C until analysis. Prior to analysis, milk samples were centrifuged for 10 min at 3500 × g at 10 °C. The upper cream layer was removed by aspirating through a Pasteur pipette. Skimmed milk was used directly in the test (100 µl per well).

2.2. Regents and equipment

AFM1 concentrations were determined by competitive enzyme immunoassay for Ridascreen “Enzyme immunoassay for the quantitative analysis of aflatoxin M1” (R1121, R-Biopharm AG, Darmstadt, Germany). The test kit contained standards and reagents: standard solutions of AFM1 in milk buffer (levels: 0, 5, 10, 20, 40 and 80 ng/L), anti-aflatoxin M1 antibody (concentrate), conjugate (peroxidase conjugated aflatoxin M1, concentrate), substrate/chromogen (tetramethylbenzidine), stop solution (1 N H2SO4), sample dilution buffer, conjugate, antibody dilution and substrate/chromogen (tetramethylbenzidine), stop solution (1 N H2SO4), sample dilution buffer, conjugate, antibody dilution and washing buffer for the preparation of 10 mM phosphate buffer (PBS, pH 7.4) contained 0.05% Tween 20.

Prior to analysis, additional preparation steps for regents included: 1:1 dilution of conjugate and antibody concentrates by the buffer; dissolution of buffer salt in 1 L of distilled water (ready for use for 4–6 weeks).

The standard stock solution of AFM1 (1000 mg/L) was prepared by dissolving the standard AFM1 (Pr. No.: A6428, Sigma–Aldrich, St. Louis, USA) in methanol (Kemika, Zagreb, Croatia). Working solutions for spiked milk samples were prepared by further dissolution of stock solution in methanol. Stock solution was stored at 4 °C for no longer than 6 months, and working solutions were prepared prior to each analysis.

The following equipment was used for sample preparation: Rotanta 460R centrifuge (Hettich GmbH & Co.KG, Tuttlingen, Germany); IKA® Vortex Genius 3 (IKA® – Werke GmbH & CO.KG, Germany). The Tecan Sunrise plate absorbance reader (Tecan Austria GmbH, Salzburg, Austria) was used for measurements of optical density at 450 nm.

2.3. Aflatoxin analysis by ELISA

Preparation of the samples and ELISA test procedure for the determination of AFM1 in milk samples was performed according to the manufacturer’s instructions. In the case of AFM1 levels higher than 80 ng/L, samples were diluted with sample dilution buffer and reanalysed.

The method was validated according the European Commission guidelines (European Commission, 2002). Validation parameters obtained were (ng/L): detection limit, LOD, 23.2 ng/L; quantification limit, LOQ, 35.8 ng/L; detection capacity, CC, 34.5 ng/L; recovery within the range 87.4–107.5% (spiked levels 25, 50 and 75 ng/L); coefficients of variation in the range 8.7–17.0%. The results indicated that the method was reliable for the determination of AFM1 in milk. Furthermore, the quality of the results was confirmed with an obtained Z value of 0.9 (acceptable range –2 ≤ z ≤ 2) in the proficiency test organized by FAPAS in milk powder samples (Proficiency Test Aflatoxin M1 04213 in 2013, Food and Environmental Research Agency, York, UK).

2.4. Statistical analyses

The results were presented as the mean ± standard deviation (SD) and also as the minimum and maximum concentrations of AFM1. Statistical analyses were performed using the Statistica 6.1 software (StatSoft®, Inc., Tulsa, USA). Analysis of variance (ANOVA) was conducted to determine differences in AFM1 concentrations in cow milk samples between eastern and other regions. A significant difference was considered significant at level of P < 0.05.

3. Results and discussion

The concentrations of AFM1 in cow, goat, sheep and donkey milk are presented in Table 1. AFM1 levels were measured in the ranges (ng/L): cow milk 3.65–162.3 (eastern Croatia) and 2.69–44.9 (other regions of Croatia); goat 2.78–40.8; sheep 2.11–5.87;

Table 1

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Range (ng/L)</th>
<th>Mean (ng/L ± SD)</th>
<th>Frequency distribution (no samples; %/AFM1 concentration (ng/L))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;23.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23.3–49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;50</td>
</tr>
<tr>
<td>Raw cow milk – Eastern part</td>
<td>194</td>
<td>3.65–162.3</td>
<td>20.6 ± 18.8*</td>
</tr>
<tr>
<td>Raw cow milk – Other regions</td>
<td>143</td>
<td>2.69–44.9</td>
<td>12.1 ± 9.49*</td>
</tr>
<tr>
<td>Raw goat milk</td>
<td>32</td>
<td>2.78–40.8</td>
<td>7.6 ± 8.94</td>
</tr>
<tr>
<td>Raw sheep milk</td>
<td>19</td>
<td>2.11–5.87</td>
<td>3.6 ± 0.91</td>
</tr>
<tr>
<td>Raw donkey milk</td>
<td>14</td>
<td>2.34–10.4</td>
<td>4.77 ± 1.83</td>
</tr>
</tbody>
</table>

* Significant difference: P < 0.001.
donkey 3.43–10.4. Concentrations exceeding the EU MRL value were found in 13 cow milk samples from eastern Croatia, with the highest level of 162.3 ng/L. AFM1 levels above the LOQ value of the method were determined in only 61 of the total 402 milk samples tested. With regard to the EU MRLs, all milk samples of goat, sheep, donkey and cow from other regions in Croatia were less than 50 ng/L. A significant difference was found between the mean AFM1 concentrations of cow milk from eastern Croatia and other regions of Croatia ($P < 0.05$). These significant differences indicate the use of contaminated feedstuff in some farms in eastern Croatia during the study period.

The results of this study were compared with the reported AFM1 concentrations in the milk of different species from other countries obtained during summer or dry seasons (Table 2). Differences in AFM1 levels found between countries were due to geographical and climate differences. To our knowledge, there are no data available for AFM1 levels in donkey milk. Therefore, the AFM1 levels measured were comparable to concentrations in the milk of other species.

Previous studies in Croatia reported similar AFM1 levels to those in cow milk from other parts of Croatia in summer in this study (Bilandzić et al., 2010; Markov et al., 2010). However, the mean AFM1 concentrations found in cow milk from eastern Croatia were similar to the mean values of 22, 20.6, 26 and 17.4 ng/L measured in summer months in recent studies from Iran, Croatia, Pakistan and Korea, respectively (Asi et al., 2012; Bilandzić et al., 2014; Lee, Kwak, Ahn, & Jeon, 2009; Nemati et al., 2010). Also, in the present study, the maximum measured concentrations of AFM1 (162.3 ng/L) in cow milk from eastern Croatia were higher than the maximum measured concentrations in those reports of 55.9, 85.9, 95 and 80 ng/L.

Mean AFM1 levels above the EU MRL were determined in Thailand and Lebanon, with 47.5 and 73.6% samples exceeding the EU MRL (Assem, Mohamad, & Oula, 2011; Ruangwises & Ruangwises, 2009). In India, Brazil and China, 48.9, 30.2 and 44.4% of total samples, respectively, were found to have concentrations exceeding the EU MRL (Picinin et al., 2013; Siddappa et al., 2012; Xiong et al., 2013). Accordingly, in tropical and subtropical countries, AFM1 occurrence is a real problem year round, including summer (Assem et al., 2011; Picinin et al., 2013; Ruangwises & Ruangwises, 2009). In these countries, conditions such as a hot and humid climate and long periods of high temperatures are conducive for the growth of toxigenic fungi. Therefore, continuous monitoring of AFM1 in milk is mandatory, especially in dry periods (Picinin et al., 2013). In Pakistan, as a subtropical country, variations and increased AFM1 levels were measured year round within the range of 199–503 ng/L (Hussain & Anwar, 2008). In Sudan and India, even higher AFM1 concentrations were obtained, in the ranges of 2070–6900 ng/L and 100–3800 ng/L, respectively (Elzupir & Elhussein, 2010; Siddappa et al., 2012).

As previously stated, literature data on AFM1 concentrations in sheep and goat milk are scarce in comparison to cow milk. In the present study, lower mean concentrations of AFM1 in sheep and goat milk samples in Croatia were determined than in the summer period in Pakistan and Iran (Asi et al., 2012; Fallah et al., 2011). Also, concentrations obtained in sheep milk were 8-times lower than those found in previous studies on sheep milk from the Istrian region of Croatia (Duraković et al., 2013). Low AFM1 concentrations measured in donkey milk also suggested and confirmed the availability of pastures and forage for animals during summer.

Elevated concentrations of AFM1 determined in milk in different countries in dry periods or during winter are influenced by the use of greater amounts of mixed supplementary feedstuff, dry hay and corn contaminated with high levels of AB1 (Asi et al., 2012; Fallah et al., 2011; Xiong et al., 2013). For example, AFM1 levels above the EU MRL were determined in the winter and spring season in Iran (Fallah et al., 2011; Nemati et al., 2010; Rahimi, Boybadian, Rafiei, & Kazemeini, 2010). A recent study conducted on maize samples in three Croatian regions showed the highest concentrations of AB1 in maize were from eastern Croatia, where 36.5% of samples had higher levels than the maximal permitted level of 20 μg/kg (Pleadin et al., 2014). Consequently, in a recent study of AFM1 in raw and UHT milk samples in Croatia during the winter—summer season of 2013, seasonal differences were determined in the total mean AFM1 levels (Bilandzić et al., 2014). Elevated AFM1 concentrations above the EU MRL of 50 ng/kg were found in 27.8% of raw milk samples and 9.64% of UHT milk samples. The highest percentage of positive samples of raw milk was found in February, March and April (45.9%, 35.4% and 29.9%, respectively). The highest AFM1 of 1135 ng/kg was determined. A gradual decline in concentrations was found in the period from May to June while not a single sample with elevated concentrations in either type of milk was found in July. The results of this study confirmed a lower incidence of elevated AFM1 levels in summer when large quantities of fresh

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**Table 2**

<table>
<thead>
<tr>
<th>Country</th>
<th>Year of sampling</th>
<th>Species</th>
<th>Seasone/month</th>
<th>Mean/Mean ± SD (ng/L)</th>
<th>Maximal measured value (ng/L)</th>
<th>Above EU MRL (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lebanon</td>
<td>2012</td>
<td>Cow</td>
<td>September</td>
<td>13.89</td>
<td>−</td>
<td>−</td>
<td>Hassan and Kassaiś (2014)</td>
</tr>
<tr>
<td>Brazil</td>
<td>2009–2010</td>
<td>Cow</td>
<td>Dry period</td>
<td>35.9 ± 4.4</td>
<td>105.7</td>
<td>30.2</td>
<td>Picinin et al. (2013)</td>
</tr>
<tr>
<td>China</td>
<td>2010–2011</td>
<td>Cow</td>
<td>Summer</td>
<td>31.9 ± 26.7</td>
<td>82</td>
<td>44.4</td>
<td>Xiong et al. (2013)</td>
</tr>
<tr>
<td>Pakistan</td>
<td>2010</td>
<td>Cow</td>
<td>Summer</td>
<td>22 ± 6</td>
<td>95</td>
<td>−</td>
<td>Asi et al. (2012)</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td>Goat</td>
<td>Summer</td>
<td>18 ± 8</td>
<td>88</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td>Sheep</td>
<td>Summer</td>
<td>24 ± 9</td>
<td>69</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Croatia</td>
<td>2007</td>
<td>Sheep</td>
<td>June</td>
<td>24</td>
<td>24–29</td>
<td>0</td>
<td>Duraković et al. (2013)</td>
</tr>
<tr>
<td>India</td>
<td>2011</td>
<td>Cow</td>
<td>Spring–summer</td>
<td>−</td>
<td>3800</td>
<td>48.9</td>
<td>Siddappa et al. (2012)</td>
</tr>
<tr>
<td>Lebanon</td>
<td>2010</td>
<td>Cow</td>
<td>Spring–summer</td>
<td>60.4</td>
<td>126</td>
<td>73.6</td>
<td>Assem et al. (2011)</td>
</tr>
<tr>
<td>Iran</td>
<td>2008</td>
<td>Cow</td>
<td>Summer</td>
<td>28 ± 5</td>
<td>−</td>
<td>−</td>
<td>Fallah et al. (2011)</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td>Goat</td>
<td>Summer</td>
<td>11 ± 4</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td>Sheep</td>
<td>Summer</td>
<td>19 ± 5</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Croatia</td>
<td>2010</td>
<td>Cow</td>
<td>Summer</td>
<td>17.4 ± 3.1</td>
<td>36.52</td>
<td>0</td>
<td>Markov et al. (2010)</td>
</tr>
<tr>
<td>Croatia</td>
<td>2009</td>
<td>Cow</td>
<td>June</td>
<td>7.5 ± 3.3</td>
<td>11.6</td>
<td>0</td>
<td>Bilandzić et al. (2010)</td>
</tr>
<tr>
<td>Iran</td>
<td>2006</td>
<td>Cow</td>
<td>Summer</td>
<td>17.4 ± 3.1</td>
<td>55.9</td>
<td>−</td>
<td>Nemati et al. (2010)</td>
</tr>
<tr>
<td>Thailand</td>
<td>2006–2007</td>
<td>Cow</td>
<td>Summer</td>
<td>50 ± 21</td>
<td>47.5</td>
<td>44.7</td>
<td>Lee et al. (2009)</td>
</tr>
</tbody>
</table>

*Not mentioned in the references.*
animal feed, such as pasture and green fodder, are available for lactating animals. However, the elevated levels of AFM1 in 13 cow milk samples from eastern Croatia clearly indicate the use of contaminated supplementary feedstuff in some farms during the study period.

4. Conclusions

In this study, AFM1 levels exceeding the LOQ value of the method were detected in only 15.2% of the total milk samples tested during the period July–September 2013. Among these, 14.7% were cow milk and only 0.5% were goat milk samples. Concentrations of AM1 in all milk samples of goat, sheep, donkey and cow from other regions in Croatia were below the EU MRL (50 ng/L). This confirmed the use of fresh pastures and forage for lactating animals in the study period, particularly in other regions of Croatia. The obtained results were similar to the literature data for AFM1 in other countries in the summer period.

However, AFM1 concentrations exceeding EU MRL values were detected in 6.7% of cow milk samples from eastern Croatia. This was expected given the elevated levels of AB1 in maize from eastern Croatia and also high AFM1 concentrations (>50 ng/L) in cow milk from the same region found in two recent studies. Therefore, these results suggest the use of contaminated feedstuff in some farms in eastern Croatia during the study period. Accordingly, feed control for dairy cattle and adequate storage conditions for feed should be established to prevent the consequent occurrence of AFM1 in milk.

References


