DETERMINATION OF OXYTETRACYCLINE RESIDUES IN MILK WITH THE USE OF HPLC METHOD AND TWO MICROBIAL INHIBITION ASSAYS

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The aim of this study was to compare the sensitivity of the disc diffusion method, the Four-plate test (FPT), and the HPLC method for the determination of oxytetracycline residues in milk. Milk samples were collected from six cows with mastitis which were intramammary treated with Oxymykoin for 5 d. Milk samples were collected and tested for the presence of OTC residues at intervals of 0, 24, 48, 72, 96, 120, 144, 168 and 192 h after the first drug administration. The highest average concentration of 195.68 mg OTC/kg was detected by HPLC method 5 d after the first drug administration (the last day of therapy). Two positive samples (zone diameters ≤ 1 mm) were detected using the microbiological assay with Bacillus stearothermophilus var. calidolactis C 953 in milk samples before the first drug administration (0 h). When the same samples were tested with the dialysis membrane, negative results were obtained. As seen from the results of this study, positive milk samples confirmed by HPLC method in which the concentrations of OTC residues exceeded the MRLs were not always detected by the agar diffusion test with Bac. stearothermophilus.

Key words: milk, oxytetracycline, HPLC, microbial inhibition assays.

Antibiotics represent the most important group of exogenous inhibitory factors in milk. Numerous antibacterial substances are used worldwide, the most common being aminoglycosides, tetracyclines, macrolides and sulphonamides. Tetracycline antibiotics are produced by Streptomyces spp. (3). Oxytetracycline (OTC) is known as a broad-spectrum antibiotic with a bacteriostatic effect on the wide range of gram-negative and gram-positive bacteria. The mode of action lies in its binding to 30S ribosomal subunits of bacteria, thus inhibiting the protein synthesis (5). In the Slovak Republic (SR), an intramammary infusion of Oxymykoin is often applied to dairy cows with mastitis. Microbiological methods used for the detection of drug residues in milk are mainly based on the inhibition of various sensitive test micro-organisms. The microbial inhibition assays routinely applied in many countries use Bacillus stearothermophilus as the test microorganism which is especially sensitive to β-lactam.
antibiotics. The maximum residual limits (MRLs) of antibiotics in food are set by the Codex Alimentarius of the Slovak Republic (14). Milk samples exceeding prescribed limits must be excluded from human consumption.

The purpose of this study was to determine the presence of oxytetracycline residues in milk from cows with clinical signs of mastitis being treated with OTC. Milk samples were tested for the OTC residues within the whole period of antibiotic intramammary treatment (5 d), as well as during four days after the last drug administration. In order to compare their sensitivity, three methods were used simultaneously for the determination of OTC residues in milk: the HPLC method, the agar diffusion test with *Bac. stearothermophilus* and the Four-plate test (FPT).

**Material and Methods**

Black-red breed cows with clinical signs of mastitis confirmed at the University of Veterinary Medicine in Košice were involved in this study. Oxymykoin (2.2 g oxytetracyclinum in 46 g of foam; 0.44 g per one quarter, Galena Opava, Czech Republic) was intramammary administered to six cows at 24-hour-intervals. The equal volumes of foremilk from each quarter of the same cow were taken during milking in the morning and in the afternoon, and then mixed together. Milk samples were collected immediately before the first drug administration (0 h), and then after 24, 48, 72, 96, 120, 144, 168, and 192 h (the withdrawal period of OTC is 5 d). The oxytetracycline residues in milk were detected using the HPLC-procedure (10) with a sensitivity of 50 µg/kg; the agar diffusion assay with *Bac. stearothermophilus* var. *calidolactis* C 953 as the test microorganism; as well as the FPT using the spores of *Bac. subtilis* BGA at three different pH-values (6.0, 7.2 and 8.0) and *Micrococcus luteus* (4). Commercially distributed test media and the spore-suspensions of sensitive microorganisms (MERCK, Germany) were used for both of the microbiological assays.

The discs of filter paper (Whatman 1, Ø 12 mm) were moistened with 0.1 ml of milk samples, and then placed on the surface of test-medium containing the spores of sensitive test microorganisms. The plates with *Bac. stearothermophilus* were then incubated at 63–65°C for 3–5 h, the plates with *Bac. subtilis* at 30°C for 18–24 h, and the plates with *Micrococcus luteus* at 37°C for 18–24 h. The positive results (the presence of OTC residues) were manifested by the formation of transparent zones around the discs.

In addition to common procedure of both microbiological assays, dialysis membrane (Visking dialysis tubing, Type C/150, SERVA, Germany) was placed between the discs and the agar medium in order to differentiate between diffusible (e.g. antibiotics) and non-diffusible inhibitory substances with antimicrobial effects.

**Results**

The results of HPLC analysis are shown in Table 1. All the milk samples showed positive results for the presence of antibiotic residues twenty-four hours after the first drug administration. However, the average Oxymykoin concentrations were lower than those determined 48 h after the first administration (59.13 ± 70.64 mg/kg versus 62.56
± 33.46 mg/kg). The highest concentration of OTC was observed 96 h after the beginning of therapy (195.68 ± 94.29 mg/kg versus 2.74 ± 4.32 mg/kg). One hundred and forty-four hours after the first drug administration, the residues of oxytetracyclines in milk were only found in four of six cows (67%) involved in the experiment. One cow excreted Oxymykoin more slowly than the other five did. The milk from this cow showed the presence of OTC residues within 3 d after the last drug administration.

Table 1
Oxytetracycline (OTC) concentrations in milk samples determined by HPLC method

<table>
<thead>
<tr>
<th>Periods after the first OTC administration (h)</th>
<th>Concentrations of OTC in milk (mg/kg)</th>
<th>Positive samples/Total number</th>
<th>Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>7.37 – 185.92</td>
<td>59.13</td>
<td>70.63</td>
</tr>
<tr>
<td>48</td>
<td>17.26 – 105.47</td>
<td>62.56</td>
<td>33.45</td>
</tr>
<tr>
<td>72</td>
<td>35.09 – 92.45</td>
<td>57.70</td>
<td>22.67</td>
</tr>
<tr>
<td>96</td>
<td>80.78 – 351.58</td>
<td>195.68</td>
<td>94.29</td>
</tr>
<tr>
<td>120</td>
<td>0.72 – 7.11</td>
<td>2.78</td>
<td>2.46</td>
</tr>
<tr>
<td>144</td>
<td>ND – 1.58</td>
<td>0.39</td>
<td>0.60</td>
</tr>
<tr>
<td>168</td>
<td>ND – 0.18</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>192</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND – not detected
SD – standard deviation

The results of both microbiological assays performed are recorded in Tables 2 and 3. At the beginning of the trial with Bac. stearothermophilus, two positive samples (zone diameters ≤ 1 mm) were detected. Twenty-four hours after the first Oxymykoin administration, five positive samples were observed. One hundred and twenty hours after the first drug administration, the number of positive samples decreased to three. The highest inhibition was noticed 72 h after the first drug administration (zone diameters between 0 and 9 mm). No inhibition was observed in milk samples 144 h after the first drug administration.

As to the results obtained with FPT, no inhibition was detected at the beginning of the trial. Using the spores of Bacillus subtilis BGA at pH 6.0, all the milk samples gave positive results 24 h after the first OTC administration. One hundred and twenty hours after the first administration, the residues were detected in none of the samples. The highest inhibition was observed 96 h after the first administration (zone diameters between 11 and 20 mm). One hundred and sixty-eight hours after the first administration, no inhibition was detected in milk samples.

When the spores of Bacillus subtilis BGA at pH 7.2 were used, four positive milk samples have been found 24 h after the first OTC administration, five positive samples 48, 72, and 96 h after the first OTC administration; and two positive samples
120 h after beginning of the therapy. No inhibition was observed in milk samples 144 h after the first drug administration.

**Table 2**
The diameters of inhibitory zones determined by microbiological assays without the dialysis membrane

<table>
<thead>
<tr>
<th>Periods after the first administration (h)</th>
<th>Bac. stearothermophilus pH 6.0</th>
<th>M. luteus</th>
<th>Bac. subtilis pH 7.2</th>
<th>Bac. subtilis pH 8.0</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Range Posit./Total (mm)</td>
<td>Range Posit./Total (mm)</td>
<td>Range Posit./Total (mm)</td>
<td>Range Posit./Total (mm)</td>
</tr>
<tr>
<td>0</td>
<td>0–2 2/6 0–2 1/6 0 0/6 0 0/6 0 0/6</td>
<td>0–2 1/6 0 0/6 0 0/6 0 0/6</td>
<td>0–2 1/6 0 0/6 0 0/6 0 0/6</td>
<td>0–2 1/6 0 0/6 0 0/6 0 0/6</td>
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<tr>
<td>24</td>
<td>0–8 5/6 0–2 2/6 4–17 6/6 2–10 4/6 0–10 3/6</td>
<td>0–2 4/6 0–2 2/6 4–17 6/6 2–10 4/6 0–10 3/6</td>
<td>0–2 2/6 0–2 2/6 4–17 6/6 2–10 4/6 0–10 3/6</td>
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<td>0–6 4/6 0–2 4/6 2–19 6/6 0–11 5/6 0–7 5/6</td>
<td>0–6 4/6 0–2 4/6 2–19 6/6 0–11 5/6 0–7 5/6</td>
<td>0–6 4/6 0–2 4/6 2–19 6/6 0–11 5/6 0–7 5/6</td>
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<tr>
<td>72</td>
<td>0–9 5/6 0–2 4/6 3–17 6/6 0–11 5/6 0–6 5/6</td>
<td>0–9 4/6 0–2 4/6 3–17 6/6 0–11 5/6 0–6 5/6</td>
<td>0–9 4/6 0–2 4/6 3–17 6/6 0–11 5/6 0–6 5/6</td>
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<tr>
<td>96</td>
<td>0–9 5/6 0–5 5/6 11–20 6/6 0–10 5/6 ND ND</td>
<td>0–9 5/6 0–5 5/6 11–20 6/6 0–10 5/6 ND ND</td>
<td>0–9 5/6 0–5 5/6 11–20 6/6 0–10 5/6 ND ND</td>
<td>0–9 5/6 0–5 5/6 11–20 6/6 0–10 5/6 ND ND</td>
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<tr>
<td>144</td>
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<td>0 0/6 0 0/6 0 0/6 0 0/6 0 0/6 0 0/6 0 0/6</td>
</tr>
</tbody>
</table>

When *Bacillus subtilis* BGA at pH 8.0 was used, three positive milk samples were detected 24 h after the first OTC administration. Fourty-eight, and 72 h after the start of therapy, five samples of milk showed the inhibition zones. The most intense inhibition was observed 48 h after the first drug administration (zone diameters between 0 and 10 mm). After 120 h, no inhibition was observed in milk samples.

Using the test with *Micrococcus luteus* ATCC 9341, two positive milk samples were detected 24 h after beginning of the therapy. The inhibition zones were observed in four milk samples after 48 and 72 h and in five milk samples after 96 h. The highest inhibition was observed 96 h (diameter zone) after the first drug administration (zone
As seen from the results, *Bac. subtilis* at pH 6.0 showed the highest sensitivity (zone diameters between 0 and 20 mm) and *M. luteus* the lowest sensitivity (zone diameters between 0 and 5 mm) to the presence of OTC residues in milk. When the dialysis membrane was used, the diameters of inhibition zones in positive milk samples decreased by 1–2 mm.

**Discussion**

Screening of milk samples for the presence of antibiotic residues is usually performed with the help of microbial inhibition assays. Their sensitivity to different drugs depends mainly on the indicator microorganism used and the concept of the test. The assays with *Bac. stearothermophilus* as the test microorganism are routinely used in milk industry worldwide. As seen from the results of this study, *Bac. stearothermophilus* is not sufficiently sensitive to the residues of oxytetracycline in milk. Positive milk samples confirmed by HPLC method in which the concentrations of OTC residues exceeded the MRLs were not always detected by the agar diffusion test with *Bac. stearothermophilus*. *Bac. stearothermophilus* is reported to be sensitive enough to the group of β-lactam antibiotics. However, its sensitivity to other antibiotics and sulphonamides (7, 9, 12) is described as insufficient. As reported, the sensitivity of *Bac. stearothermophilus* to tetracycline ranges between 400 and 500 µg/kg (6). The *Codex Alimentarius* of the Slovak Republic set the MRLs for tetracyclines in milk to 0.1 mg/kg.

The lower detection limits ranging from 0.1 to 0.2 µg/ml are described (11) for the titration test with *Bacillus cereus* var. *mycoides* ATCC 9634 and TTC (triphenyltetrazolium chloride). The *Bac. cereus* test with an indicator is the modification of the Six-plate-test (6). Its sensitivity can be increased by the pre-treatment of samples with ammonium oxalate (11). The detection limits (µg/kg) for tetracyclines (oxalate pre-treatment in parentheses) are reported as follows: tetracycline 100 (60); chlorotetracycline 60 (20); oxytetracycline 100 (70); doxycycline 60 (40), and rolitetracycline 0 (80).

In the last few years, a new microbiological assay (the Tet-Lux test) has been developed for the detection of tetracycline residues in raw milk (8). It uses *Escherichia coli* bacteria carrying a sensor plasmid, in which a tetracycline-specific control unit regulates the expression of bacterial luciferase genes. The presence of tetracycline residues in the sample causes an increase in the light emission of the test bacteria. This assay is able to detect 4–35 ng/ml of tetracycline, oxytetracycline, chlorotetracycline, doxycycline, demeclocycline, methacycline, and minocycline.

As recorded in Tables 2 and 3, two positive samples (zone diameters ≤ 1 mm) were detected by the test with *Bac. stearothermophilus* var. *calidolactis* C 953 in milk samples at the beginning of the trial before the first OTC administration to cows with mastitis. When tested the same samples with the dialysis membrane, negative results were obtained. As known, the amount of both the natural inhibitors lysozyme and lactoferrin is often abnormally high in colostrum, as well as in milk from cows with mastitis. The latest may even contain 3 µg/ml of lysozyme and 8 µg/ml of lactoferrin,
these compounds being responsible for false-positive results of microbiological residue assays both alone and in a combination (1, 2, 13).

References