A Plate Diffusion Method for Detecting Fluoroquinolone Residues in Raw Cow’s Milk

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Abstract


The plate diffusion method is a reference method in the Czech Republic for determination of residues of antimicrobial agents in raw materials and foodstuffs of animal origin. A new method using the E. coli strain ATCC 11303 for the detection of fluoroquinolones was introduced in 2008. The aim of this study was to determine the detection capability (CCβ) of this modified method using this E. coli strain for selected fluoroquinolones registered in the Czech Republic for treating diseases in cattle – danofloxacin, marbofloxacin, ciprofloxacin, enrofloxacin, and flumequine. When comparing the maximum residue limits for individual fluoroquinolones and the CCβ values determined, we can state that the method displays very good sensitivity to ciprofloxacin and enrofloxacin (20 and 40 µg/l), marbofloxacin (70 µg/l), and danofloxacin (30 µg/l). The CCβ of the method for flumequine was not found in concentrations ≤ MRL. The method did not display sensitivity to flumequine even in a concentration equal to twelve times the MRL.

Keywords: residues; milk; quinolones; microbiological method

Monitoring for the presence of residues of antimicrobial agents in raw milk is, according to the current legislation, one of the most important indicators of milk quality. It is important both in technological terms and, first and foremost, from the viewpoint of ensuring the health safety of milk and milk products.

The widespread use of antimicrobial agents in livestock is accompanied by the risk of the occurrence of residues of these agents in products of animal origin. Residues of veterinary medicines in raw materials and foodstuffs of animal origin may represent a potential health risk for consumers. Screening methods are used in practice for the detection of residues of veterinary medicines in raw materials and foodstuffs of animal origin. The purpose of these methods is to determine the presence/absence of residues of antimicrobial agents in a sample in respect of the stipulated maximum residue limit (MRL). MRL is defined as the maximum concentration of a residue of a pharmacologically active substance which may be permitted in food of animal origin. “Residues of pharmacologically active substances” means all pharmacologically active substances, expressed in mg/kg or µg/kg on a fresh weight basis, whether active substances, excipients or degradation products, and their metabolites which remain in food obtained from food-producing animals (Regulation 470/2009). Microbiological inhibition methods play an important role in the system of monitoring residues of antimicrobial agents. Plate diffusion methods are a significant part of this group of methods. They remain important screening and post-screening methods today as they are capable of detecting a broad spectrum of antibacterial agents with various chemical structures (Botsoglou & Fletouris 2001). A number of studies considering plate diffu-
tion methods for detecting residues of antimicrobial agents in raw materials of animal origin have been conducted and published in recent years with the aim of increasing the sensitivity of these methods, determining detection limits for various antibiotics, developing new multi-plate systems, applying new test strains, and comparing the sensitivity of the given methods with the demands of the current legislation (Aureli et al. 1996; Nouws et al. 1999a,b; Okerman et al. 2001; Lee et al. 2007; Althaus et al. 2009; Pikkemaat 2008).

Antimicrobial agents represent an extensive and dynamic group of substances in veterinary medicine that is continually growing with the development of new preparations. In order to ensure the health safety of raw materials and foodstuffs of animal origin, it is important to introduce new test methods capable of detecting the widest possible spectrum of antimicrobial agents that are sensitive to concentrations ≤ MRL. Fluoroquinolones represented considerable progress in expanding the spectrum of medicines for effective treatment of infectious diseases. At the present time, fluoroquinolones are classified according to the OIE as belonging to the group of Critically Important Antimicrobials for Veterinary Use (FAO 2007).

The plate diffusion method is a reference method for determining residues of antimicrobial agents in raw materials and foodstuffs of animal origin in the Czech Republic (State Veterinary Administration 2008). A new plate diffusion method with a strain of E. coli displaying sensitivity to fluoroquinolones has been developed in line with the STAR method (Gaudin et al. 2004), the official method recommended by the reference laboratory of the European Community (Star Protocol 2002). The aim of this study is to determine the detection capability (CCβ) of the modified method with E. coli ATCC 11303 for fluoroquinolones registered in the Czech Republic for the treatment of diseases in cattle: danofloxacin (DANO), marbofloxacin (MARBO), ciprofloxacin (CIPRO), enrofloxacin (ENRO), and flumequine (FLU).

MATERIAL AND METHODS

Standard solutions of fluoroquinolones. The following analytic standards were used for the preparation of standard solutions of fluoroquinolone chemotherapeutics: CIPRO (17850, Fluka), ENRO (17849, Fluka), FLU (F 7016, Fluka), DANO (3377, Riedel-de Haën), MARBO (34039, Riedel-de Haën) from the company Sigma Aldrich (St. Louis, USA). Standard solutions of a concentration of active substance of c = 1 mg/ml were prepared by dissolving a charge of the standard in 1 ml NaOH (c = 0.1 mol/l) and subsequently diluting with distilled water. Working solutions were prepared from standard solutions by diluting with distilled water.

Preparation of fortified samples of milk. Fortified samples of milk were prepared by diluting the calculated quantity of a working solution of a chemotherapeutic with milk, which was also tested for the presence of fluoroquinolones as a negative control. The range of concentrations of fluoroquinolones in the fortified samples is given in Table 1.

Plate method with the E. coli test strain ATCC 11303. A suspension was prepared from the 24-hour culture of a strain on inclined TSA (TSA, M593; Hi-Media, Mumbai, India). This suspension was added to test agar pH 8 (Antimicrobial Inhibitor Test Agar pH 8.0; M 1632; HiMedia, Mumbai, India) cooled to a temperature of 45–48°C. The concentration of bacterial cells in the agar was approximately 10⁵ CFU/ml. Sterile Petri dishes of 90 mm in diameter were filled with 4 ml of agar. The sensitivity of the method was monitored by a test 10 µl working solution of CIPRO (c = 0.3 µg/ml) on a disc of 6 mm in diameter.

Determination of detection capability. Fortified samples of milk were stirred and applied (100 µl) to a disc of 12.7 mm in diameter (Blanc paper discs; Albet® LabScience, Barcelona, Spain). Each fortified sample with basic concentrations of antibiotic equal to 0.5, 1, and 1.5 times the MRL was tested twenty times. The fortified samples with further concentrations of antibiotics for more specific designation of the detection capability level were tested sixty times. 60 blank samples were also analysed at the same time and the false negative rate was determined (Table 1). The plates were incubated at 37°C for a period of 18–24 hours. The complete inhibition of the strain growth around the disc – the inhibition zone (IZ) – was evaluated following the completion of incubation, and its size in mm was recorded. The width of the IZ is defined as the distance of the edge of the disc. The size of the IZ was measured using a special scale (HiAntibiotic Zone Scale; HiMedia, Mumbai, India). A regular IZ of a size ≥ 2 mm was interpreted as a positive (suspect) test result (Star Protocol 2002; State Veterinary Administration 2008).

Plate sensitivity was stipulated in accordance with the requirements of the European Union legislation (Commission Decision 657/2002) for the working
characteristics of screening methods on the basis of detection capability ($CC\beta$). $CC\beta$ is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of $b$. In the case of analytes with an established MRL, $CC\beta$ is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of $1-\beta$. $CC\beta$ is the concentration at which only $\leq 5\%$ false compliant results remain. In this case, $CC\beta$ must be lower than or equal to the MRL.

RESULTS AND DISCUSSION

The plate diffusion method with an $E. \ coli$ test strain presented here differs from the STAR (Screening Test for Antibiotic Residues) method in methodical terms. The discs used for the application of the studied milk sample are of different size. The STAR method recommends discs of a size of 9 mm, while discs of 12.7 mm in diameter are used in accordance with the methodical directive of the State Veterinary Administration. Another difference consists in the amount of agar in a Petri dish. The STAR method recommends 5 ml of agar in a Petri dish, in our study we used 4 ml. A regular I2Z of a size ≥ 2 mm is considered a positive (suspect) test result on a plate with a strain of $E. \ coli$ by both methodologies (State Veterinary Administration 2008; Star Protocol 2002). The sensitivity of plate diffusion methods to antimicrobial agents may be affected by a number of factors: methods of sample application, the height of the agar in a Petri dish, the test medium, the pH of the medium, the sensitivity of the test strain, the number of CFU of the strain in 1 ml of test medium, physical and chemical properties and composition of the matrix, etc. (Botsoglou & Fletouris 2001; Gaudin et al. 2010).

The European Union legislation stipulates the following MRL values for fluoroquinolones in raw cow’s milk: 30 µg/kg for DANO, 50 µg/kg for FLU, 75 µg/kg for MARBO and 100 µg/kg as the sum of residues of ENRO and CIPRO (Commission Regulation 37/2010).

<table>
<thead>
<tr>
<th>Fluoroquinolone</th>
<th>MRL EU (µg/kg)</th>
<th>$CC\beta$ (µg/l)</th>
<th>False-negative rate</th>
<th>$CC\beta$/MR</th>
<th>Range of spiked milk concentrations (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danofloxacin</td>
<td>30</td>
<td>30</td>
<td>60 +</td>
<td>$CC\beta = MRL$</td>
<td>15–45</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>75</td>
<td>70</td>
<td>60 +</td>
<td>$CC\beta &lt; MRL$</td>
<td>37.5–112.5</td>
</tr>
<tr>
<td>Flumequine</td>
<td>50</td>
<td>&gt; 600*</td>
<td>60 +</td>
<td>$CC\beta &gt; MRL$</td>
<td>25–600</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100</td>
<td>20</td>
<td>60 +</td>
<td>$CC\beta &lt; MRL$</td>
<td>10–150</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>100</td>
<td>40</td>
<td>60 +</td>
<td>$CC\beta &lt; MRL$</td>
<td>10–150</td>
</tr>
</tbody>
</table>

*The method did not display sensitivity even at a concentration twelve times the MRL > 600 µg/l.
sensitive enough, the results of Gaudin et al. (2004) indicated that the sensitivity of the plate method with the *E. coli* strain according to the STAR protocol is better.

A number of other studies have also focused on the sensitivity of plate diffusion methods to various antimicrobial agents during the testing of raw milk. Chung et al. (2009) determined the sensitivity of a plate diffusion method with the *E. coli* strain ATCC 11303 (pH 8) prepared according to the STAR method for ENRO and CIPRO, although they used discs of 10 mm in diameter to test samples of milk. The minimum detectable concentrations (MDC) of the method were 20 µg/l for ENRO and 10 µg/l for CIPRO. According to the authors, the STAR protocol is capable of detecting a low level of quinolone-type antibiotics such as ENRO and CIPRO in milk. Results of their study show that the authors found the same sensitivity of *E. coli* strain to quinolones ENRO and CIPRO as published by Gaudin et al. (2004).

Nouws et al. (1999a) described a multi-plate system comprised of seven plates for the detection of residues of various antimicrobial agents in milk. The estimated detection levels for ENRO, FLU, and MARBO on a plate with *E. coli* ATCC 11303 (pH 6) were 4, 150, and 5 µg/l, respectively. A different method for the application of milk samples was used in this study. The height of the agar in a Petri dish was 2.2 mm and a milk sample of 250 µl in volume was pipetted into holes in the agar of 14 mm in diameter. The method of application of a sample may have a significant effect on the sensitivity of the method. Certain methods of application make it possible to test larger volumes of liquid samples (the use of cylindrical metal rollers, the creation of holes in the medium), which in turn means that the sensitivity of the method can be increased. This method did not display sufficient sensitivity to FLU or colistin.

Nouws et al. (1999b) evaluated the suitability for confirmation of the presence of antibiotic residues in milk. The estimated detection levels of the method with *E. coli* (RIK 144, pH 6) for ENRO, FLU and colistin were < 10, 150, and 100 µg/l, respectively. They stated that residues of quinolones could be detected in milk using this method with *E. coli* at levels ≤ MRL.

Although the plate method with an *E. coli* strain used in the Czech Republic differs from the published studies in methodical terms, we can state that the results of our study (similarly to other studies) demonstrate very good sensitivity of the test strain of *E. coli* to the fluoroquinolones tested (≤ MRL), with the exception of FLU.

The majority of the multi-plate diffusion methods for detecting residues of antimicrobial agents in various matrices (milk, meat, organs) contain a plate with a strain of *E. coli* for the detection of quinolones. These methods use various strains of *E. coli* and substrates of various pH, e.g. *E. coli* Bayer 14 at pH 6 (Okerman et al. 2001), *E. coli* RIK 144 at pH 6 (Nouws et al. 1999b), *E. coli* ATCC 11303 at pH 7.2 (Myllyniemi et al. 2001) and/or at pH 8 (Gaudin et al. 2004). According to Choi et al. (1999), the *E. coli* strain ATCC 128 was highly suitable as an indicator of microorganisms in a microbial inhibition test for selective detection of fluoroquinolone residues in animal tissues.

The sensitivity of the method may also be influenced by the pH of the agar. The study has evaluated and compared the sensitivity of the method with a strain of *E. coli* to quinolones and differences in the sensitivity of the medium at pH 6 and 8. The plate with *E. coli* at pH 8 was more sensitive and detected 5 quinolones out of 8 at levels ≤ MRL in poultry meat (Okerman et al. 2007).

The given method, as was shown again by our study as well as other studies, is not sufficiently sensitive to certain quinolone chemotherapeutics, for example flumequine and oxolinic acid. Pikkemaat et al. (2008) perfected a plate diffusion method for detecting residues of antimicrobial agents in the tissues of slaughtered animals by replacing the strain of *E. coli* used in the method with *Yersinia ruckeri* NCIM 13282 at pH 6.5. The CCβ of the method was ≤ MRL for all the tested quinolones, including flumequine.

**CONCLUSIONS**

Screening methods are understood to be methods serving to detect the presence of a substance or a group of substances at a maximum residue limit. The modified plate diffusion method presented here with the *E. coli* strain ATCC 11303 displayed a CCβ at the required levels of ≤ MRL for all the tested fluoroquinolones except FLU. This is a significant finding in view of the fact that the method is part of a multi-plate method with predicted sensitivity to a significant group of antimicrobial agents.

**References**


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