Testing of Raw Milk for Tetracycline Residues

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ABSTRACT

A newly improved Bacillus calidolactis tube diffusion test and two postscreening test systems—a receptor assay (Charm HVS; Charm Sciences, Inc., Malden, MA) and a newly developed Bacillus cereus ATCC 11778 mycoides test system—were evaluated for the detection and identification of tetracycline residues using 973 samples of bulk milk taken at random in The Netherlands. All milk samples were assayed with the B. calidolactis tube and the receptor test. The milk samples testing as suspect or positive with one or both of the test systems were analyzed with HPLC (limit of detection, 10 ng/ml) and the recently developed B. cereus test system.

The B. calidolactis tube diffusion test detected tetracycline residues >45 ng/ml in milk. With the B. cereus test plate, residues of oxytetracycline and tetracycline of >30 ng/ml milk were detected; for chlortetracycline and doxycycline, the detection limit was 10 ng/ml. Raw milk exhibiting inhibition diameters of <20 mm on the B. cereus test plate fulfilled the European Union criterion for maximum residue level for tetracyclines (including their 4-epimer derivatives) of <100 ng/ml (including their 4-epimer derivatives). The detection limits of the receptor assay depended on the type of milk used. The scintillation counts that were obtained for control samples of bulk milk were considerably lower than for the milks obtained from Charm Sciences, Inc. or processed using UHT pasteurization.

One of 973 milk samples was suspect for tetracycline residues by means of the B. calidolactis tube test as well as by the receptor assay; 8 other samples were also considered to be positive using the receptor assay alone. The presence of tetracycline residues could not be proved for these 9 samples (residue concentration, <10 ng/ml) with HPLC. We concluded that the receptor assay was not reliable to detect tetracycline residues in raw milk at <150 ng/ml. The B. cereus test plate was determined to be an inexpensive, reliable alternative for the receptor assay for detection of tetracycline residues.

(Key words: residues in milk, tetracycline, Charm HVS test, bioassay)

Abbreviation key: EU = European Union, MAS = multiple-antimicrobial drug standards, MRL = maximum residue level.

INTRODUCTION

For the detection of antimicrobial residues in milk, microbiological screening tests are used that utilize bacterial test strains such as Bacillus stearothermophilus var calidolactis, Streptococcus thermophilus, and Bacillus subtilis ATCC 6633 (5, 8). To detect tetracycline residues, the maximum residue level (MRL) for oxytetracycline, tetracycline, and chlortetracycline residues (including their 4-epimer derivatives) in milk has been established in the European Union (EU) at 100 ng/ml (EU regulation 281/96). The sensitivity of test systems based on B. stearothermophilus and S. thermophilus does not fulfill the EU MRL detection criteria for tetracyclines. Recently, the Dutch tube diffusion test was improved for its detection of tetracycline residues in raw milk at concentrations near the MRL (detection limit, 40 to 60 ng/ml) (7).

Identification and quantification of tetracycline residues can be accomplished by various methods of HPLC and mass spectrometry (3, 6). For identification as a group, the Charm HVS test (Charm Sciences Inc., Malden, MA), a multiple-residue receptor assay for drugs in milk (3, 4, 6), can be performed. Charm HVS is based on the competition between a radiolabeled antimicrobial drug (tracer) and a drug residue in the milk for a binding reagent (microbial or immunochemical receptor). When the binding reagent of tetracycline (antibody) is added to milk that is contaminated with tetracycline residues, the tetracycline binds to the receptors, which prevents the labeled tetracycline from binding completely to these sites. Thus, the more labeled tetracycline that is bound, the less labeled tetracycline...
residue is left in the samples; these residues can be measured with a liquid scintillation counter in counts per minute. A significant decrease in the scintillation counts indicates the presence of certain drugs. Moats et al. (6) and Carson et al. (3) demonstrated that the receptor assay detected tetracycline residues in milk that were far below the FDA concern of 30 μg/kg; the practical consequence has been the unnecessary disposal of bulk milk.

Recently, a multiple-plate system has been developed for the detection and identification of various antibiotics near and above their MRL. For tetracyclines, a Bacillus cereus ATCC 11778 test system was developed that exhibits a detection limit of 10 to 30 ng/ml of milk.

The objective of this study was to evaluate the newly improved (Dutch) B. calidolactis tube diffusion test and two postscreening test systems, the Charm HVS test and the B. cereus test system, for the detection and identification of tetracycline residues.

MATERIALS AND METHODS

Standards

Pure drug standards (oxytetracycline, tetracycline-HCl, chlortetracycline, and doxycycline) with certificate of analysis were used for the preparation of stock solutions. Tetracyclines were dissolved in 0.1 M HCl. Working solutions were prepared from these standard solutions by serial dilutions in sterile UHT milk and in raw bulk milk. The stock solution of chloramphenicol (Sigma C-0378; Sigma Chemical Co., St. Louis, MO) was dissolved in methanol, and the working solution of 20 μg/ml was prepared in distilled water.

Sample Collection

Samples of bulk milk (total 973 samples) were obtained from the Milk Control Station (Zutphen, The Netherlands) from January to May 1996 (about 200 samples/mo) that originated from throughout The Netherlands. The milk samples were kept refrigerated (about 2 to 6°C) between sampling and analysis. Prior to handling, the samples were heated at 80°C for 10 min to inactivate natural inhibitory substances (lysozymes) in the raw milk.

Screening Tests

The newly improved Bacillus calidolactis tube diffusion test at pH 7 and 8 was performed as described (7) on fortified milk and survey samples. Milk standards that were fortified with tetracyclines were incorporated to establish the detection limit of the assay. Positive survey samples were reexamined by the addition of penicillinase and p-aminobenzoic acid for the residue detection of β-lactams and sulfonamides, respectively (8).

Postscreening Test Systems

B. cereus ATCC 11778 test plate. After Standard II Nutrient Agar (Merck, Darmstadt, Germany) that had been supplemented with 0.1% KH₂PO₄ was autoclaved, the medium was cooled to about 50°C, chloramphenicol was added (0.5 μg/ml of agar), and the pH was adjusted to 6.0. The mixture was inoculated with spores of B. cereus var mycoides ATCC 11778 (10⁵ spores/ml of agar), mixed well, and poured onto test plates of 24.5 × 24.5 cm (Gibco Europe Ltd., Breda, The Netherlands). The agar depth was 2.2 mm. After solidification of the agar, holes (i.d. 14 mm) were punched out of the agar, filled with 250 μl of a milk sample (blank samples, fortified milk standards, or survey samples), and followed by pipetting 25 μl of 20 μg chloramphenicol/ml of solution into each punch hole. After overnight incubation at 30°C, the inhibition diameters were measured with a vernier caliper (Digimatic, Mitutoyo Ltd, Ede, The Netherlands).

Charm HVS test system (receptor assay). The tablet reagents, zero control, and EU MRL multiple-antimicrobial drug standards (MAS) were obtained from Charm Sciences, Inc. (batch no. 005 B). The performance of the Charm HVS test on experimental, fortified, and standard control samples was determined according to instructions of the manufacturer. The MAS was dissolved in 50 and 100 ml of raw milk, Charm milk, or UHT milk. According to the criteria of the manufacturer, a milk sample must be considered suspect or positive (99% confidence) if the scintillation counts are <1911 (see Table 2); the declared scintillation counts for the MAS were 922.

HPLC. Following solid-phase extraction of suspected milk samples with a preconditioned C₁₈ cartridge, HPLC was performed on the obtained extracts for quantification of oxytetracycline and its 4-epimer, tetracycline, chlortetracycline and its 4-epimer, and doxycycline residues. The limit of quantification of the HPLC method for all these tetracycline analogues ranged from 10 to 20 ng/ml of milk.

RESULTS

Detection Limits of the Test Methods

Screening test. The detection limit of the improved B. calidolactis tube test at pH 7 (7) for oxytetracycline, tetracycline, chlortetracycline, and
doxycycline, based on 8- to 18-fold replicate analysis of the fortified samples, ranged between 40 and 45 ng/ml of milk, which was half of the EU MRL for tetracyclines of 100 ng/ml of inclusive 4-epimer derivatives.

**B. cereus test plate.** The detection limit of the *B. cereus* test plate based on fortified milk samples was =30 ng/ml for oxytetracycline and tetracycline·HCl and =10 ng/ml for chlortetracycline and doxycycline.

Table 1 shows that raw milk exhibiting inhibition diameters of <21 mm at the *B. cereus* test plate fulfills the EU MRL residue requirement for tetracyclines of <100 ng/ml (*P* < 0.05).

**Charm HVS test.** The baseline scintillation counts per minute of the blank UHT milk, raw milk, and the zero control samples (Charm milk) varied widely from one another.

Based on a 99% confidence interval (mean scintillation count of the blank minus three times the standard deviation), blank bulk milk samples may exhibit scintillation values near those of MAS and have to be considered positive according to the instructions of the manufacturer.

Table 3 shows the discrepancies between a positive result for tetracycline residues using Charm HVS and reference samples based on UHT milk, raw bulk milk, or MAS milk. The scintillation counts for these samples of milk with respect to the MRL (99% confidence interval) ranged from 1026 for raw milk to 1942 for UHT milk. The UHT milk is not suitable for use as a reference control sample for Charm HVS tests. Based on the scintillation value of the MAS reference sample, false-positive results with the Charm HVS test can be anticipated in tests of samples of raw bulk milk exhibiting oxytetracycline residue concentrations <50 ng/ml.

**Screening**

Table 4 shows that 9 of 973 (0.92%) samples of raw bulk milk were positive according to the criteria of the Charm HVS test established for the zero control standard (<1185 cpm; Table 2). One of the 9 samples was also determined to be suspect by the *B. calidolactis* tube test. The *B. cereus* test plate revealed negative results for the 973 samples (including the former 9 samples; detection limit for tetracyclines was 10 to 30 ng/ml of milk).

**Confirmation**

An HPLC analysis of the 9 milk samples that were tested positive by the Charm HVS test for tetracycline residues revealed negative results. No parent oxytetracycline (or 4-epimer derivative), tetracycline, chlortetracycline, or doxycycline residues could be detected in the 9 milk samples (Table 4).

**DISCUSSION**

The newly improved *B. calidolactis* tube diffusion test proved to be reliable and reproducible in its...
TABLE 4. Comparison of other test methods on milk samples showing positive for tetracycline with Charm HVS1 test.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Improved B. calidolactis</th>
<th>B. cereus test plate</th>
<th>Charm HVS test</th>
<th>HPLC</th>
<th>Judgment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pH 8</td>
<td>pH 7</td>
<td>Charm HVS test</td>
<td>HPLC</td>
<td></td>
</tr>
<tr>
<td>0247</td>
<td>–</td>
<td>–</td>
<td>975</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>0416</td>
<td>–</td>
<td>–</td>
<td>1015</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>0486</td>
<td>–</td>
<td>–</td>
<td>801</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0579</td>
<td>–</td>
<td>–</td>
<td>753</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0585</td>
<td>–</td>
<td>–</td>
<td>783</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0601</td>
<td>–</td>
<td>–</td>
<td>806</td>
<td>100</td>
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<td>0609</td>
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<td>–</td>
<td>844</td>
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<tr>
<td>0724</td>
<td>–</td>
<td>–</td>
<td>1145</td>
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<td></td>
</tr>
<tr>
<td>0896</td>
<td>–</td>
<td>–</td>
<td>749</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

1Charm HVS (Charm Sciences Inc., Malden, MA).
2Concentration equivalent to oxytetracycline; cpm = scintillation counts per minute.
3Results: – = negative, ± = suspected, — = <30 ng of oxytetracycline/ml, and . . . = <10 ng of oxytetracycline/ml.

TABLE 3. Detection limits of the receptor assay (Charm HVS1) for tetracyclines based on scintillation counts of control UHT milk, raw bulk milk, and multiple-antibiotic standard (MAS).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Detection limits (ng/ml)</th>
<th>3× SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>1</td>
<td>75</td>
</tr>
</tbody>
</table>

1Charm HVS (Charm Sciences Inc., Malden, MA).
2European Union maximum residue level including 4-epimer derivative.

The detection of tetracycline residues in milk. The detection limits for oxytetracycline, tetracycline, chlor-tetracycline, and doxycycline ranged from 40 to 45 ng/ml, which was half the EU MRL. One of 973 raw bulk milk samples (Sample 0247) was suspect for the presence of tetracycline residues (negative penicillinase and p-aminobenzoic acid test; negative for the presence of macrolide and aminoglycosides with Charm HVS test). The presence of tetracycline residues could not be confirmed by the B. cereus test plate or by HPLC (detection limit 10 ng of oxytetracycline/ml), but the Charm HVS test for tetracyclines was positive.

The 4-epimer derivatives of oxytetracycline, tetracycline, and chlortetracycline were not detectable by the B. calidolactis test because these derivatives are microbiologically inactive. Small quantities of the 4-epimer derivatives may be detected in edible tissues (and presumably milk), partly as the consequence of an artifact in the solid-phase extraction and clean-up procedures required for HPLC analyses of the former sample specimen (1) or by the presence of the 4-epimer derivatives in preparations used in veterinary practice. For a microbiological test, no extraction is needed, and, thus, there is no artificial formation of 4-epimer derivatives. The positive results of the Charm test could not to be related to the presence of 4-epimer derivatives because HPLC analysis of the suspected milk samples revealed concentrations <10 ng/ml for oxytetracycline and chlortetracycline (Table 4).

In a collaborative study (4), based on fortified milk samples, the receptor assay was shown to be reproducible and the incidence of false positives was about 3%; no assay of bulk milk samples (including confirmation) was performed. Our assay of random samples of bulk milk for tetracycline residues revealed an incidence of 0.92% false-positives with the receptor assay. The cause of the false positives of the receptor assay for tetracyclines may be related to the fatty acid content of raw milk samples (2). Nevertheless, the practical consequence of the acceptance of the Charm HVS results of <150 ng/ml would be the unnecessary and unjustifiable disposal of milk, which is unacceptable to producers. Also, Carson et al. (3) expressed serious doubts about the adequacy of the Charm HVS test to detect tetracycline residues of <30 ng/ml. Our study shows that tetracycline residues of <150 ng/ml found with the Charm test, must be considered with
great caution. Moreover, UHT and Charm reference milks are not suitable for use as reference control samples for Charm HVS tests for tetracycline (Table 3).

These data show that the confirmation of the presence of tetracycline residues in milk can be appropriately done with a *B. cereus* test plate. The test method is inexpensive and reliable. For inhibition diameters of <21 mm, the concentration of tetracycline residues will be below the established MRL of 100 ng/ml (*P* < 0.05). However, for safety with respect to 4-epimer derivatives, a decision criterion for the disposal of milk may be an inhibition diameter of 20 mm. The *B. cereus* test plate is part of a multiple-plate system, which is suitable for use in judging the residue state of milk samples at the established MRL for *β*-lactams, tetracyclines, macrolides, aminoglycosides, sulfonamides, and quinolones.

**ACKNOWLEDGMENTS**

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**REFERENCES**