Performances of Antibiotic Screening Tests in Determining the Persistence of Penicillin Residues in Ewe’s Milk

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ABSTRACT

Milk collected at 12-h intervals throughout 6 days from three groups of Manchega ewes (n = 12 per group), treated intramuscularly with β-lactams (benzyl penicillin procaine, ampicillin, and amoxicillin, respectively), was evaluated for antibiotic residues persistence with four microbial inhibitor tests (BRT MRL, CH ATK P&S, Delvotest SP, and Eclipse 100ov) and one enzymatic test (Penzym 100). Antibiotic depletion time was established using a logistic regression model. A clear effect (P < 0.0001) of milking order on the response of all tests was observed with the three antibiotics, but no significant effects were found for milk yield. Except with Eclipse 100ov, positive tests were observed after the recommended withdrawal period of benzyl penicillin procaine (five milking) from 2% (Delvotest SP) to 11% (CH ATK P&S). There were almost no positive responses beyond the withdrawal period (six milking) of ampicillin, except for the Penzym 100 test (7%). Residues of amoxicillin were found to persist beyond the six milking established as the withdrawal period, from 2.8% (Eclipse 100ov) to 72.4% (CH ATK P&S) of positive cases. Higher frequencies of doubtful cases were found with BRT MRL and Delvotest SP assays with the three β-lactams. Positive and doubtful results could be obtained when milk samples from individual ewes were analyzed using BRT MRL, CH ATK P&S, Delvotest SP, and Penzym 100 tests, even if farmers follow the antibiotic withdrawal periods.

The Mediterranean Basin is an important producer of milk from ewes and goats. Most of the ewe’s milk production from this area is used in cheese manufacture, often as raw milk.

As intensification of milk production in small ruminants has increased of late, the use of antimicrobial substances in dairy ewes has become the usual practice in veterinary medicine to treat mastitis and other diseases. This use could have serious consequences in public health and dairy product quality; antibiotic residues in milk might provoke antibiotic resistance (18, 33) or allergies in consumers (29) and cause defects in several fermented products (24, 30), among other problems.

To avoid the aforementioned risks, the European Union (EU) has passed Council Directive EEC 2377/90 (10), and subsequent amendments (100), establishing the maximum antibiotic residue levels in animal food. Therefore, the withdrawal periods are calculated according to these maximum residue limits (MRLs), taking into account several factors, such as animal species, administration route, dosage, and so on (8, 19).

The most important way to guarantee residue levels in milk below the established MRLs is by respecting holding periods after antibiotic treatment. The farmer’s responsibility is essential at this point to maintain the safety of any animal food produced (11). However, because there are few specific available antibiotic treatments for dairy ewes compared to those for cows, the withdrawal times have not been well established in this species (17).

In order to determine the presence of antimicrobial residues in milk and to provide toxicological and technological safety, several rapid screening methods have been developed to test milk on the farm (16, 21, 25). These methods should be used as the first step to detect antimicrobial residues, as is recommended by the integrated system for detection of antimicrobials and inhibitors in milk (20).

Most of these screening tests have been commercially available for cow’s milk. However, there are currently several screening tests for evaluating ewe’s milk, e.g., BRT (4) or Penzym (3, 27), which give highly sensitive results in the detection of antimicrobial residues in milk for this species. The detection limits of some of these methods for ewe’s milk are summarized in Table 1.

Microbial inhibition tests have been employed to evaluate the antibiotic depletion time in dairy cattle (9, 23, 31, 34). In dairy sheep, there are previous studies about the effect of several different antibiotic treatments or administration routes (5, 26) on the response of some microbial inhibition tests. However, the behavior of several different methods throughout the depletion time has not been studied.

For this reason, the aim of this study was to analyze the presence of residual antibiotics in milk after treatment of ewes with different penicillins by several recommended screening methods. An attempt has also been made to determine whether ewe’s milk could show signs of residual

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TABLE 1. Detection limits (µg/kg) of β-lactam antibiotics tested by various methods in ewe’s milk

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>BRT Delvotest SP</th>
<th>Eclipse 100ov</th>
<th>Penzym 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>6</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>51</td>
<td>23</td>
<td>—</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>2</td>
<td>1.4</td>
<td>5</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>230</td>
<td>63</td>
<td>86</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>1,330</td>
<td>610</td>
<td>—</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>270</td>
<td>68</td>
<td>115</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>92</td>
<td>41</td>
<td>107</td>
</tr>
<tr>
<td>Cefiofur</td>
<td>120</td>
<td>59</td>
<td>—</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>69</td>
<td>41</td>
<td>85</td>
</tr>
<tr>
<td>Reference(s)</td>
<td>4</td>
<td>7</td>
<td>6, 28</td>
</tr>
</tbody>
</table>

penicillin even after fulfilling the manufacturers’ recommended withdrawal period.

MATERIALS AND METHODS

Animals. Thirty-six healthy Manchegan-bred ewes (body weight range 55 to 80 kg, age from 3 to 7 years) from the Manchegan herd in the experimental flock of the Instituto Técnico Agronómico Provincial (Diputación de Albacete, Albacete, Spain) were selected for this study. None had a previous history of clinical mastitis, and all ewes presented somatic cell counts (SCC) of <100,000 somatic cells per ml before starting the treatment. The ewes were randomly divided into three groups, each containing 12 animals roughly equal in body weight, age, and daily milk yield. The animals received no drugs before the experiment was carried out. The ewes were housed and fed the same as the rest of the herd. Lambing took place in January and February, and mothers suckled their lambs for 35 days. After weaning, the ewes were machine-milked twice a day. The experiment took place in the fourth week of the milking period.

Antibiotic treatment and sample collection. Antibiotics were administered immediately after milking was completed. Each group of 12 animals was treated with two intramuscular injections of a different antibiotic at 48-h intervals. The first group was given 15,000 IU/kg body weight of benzyl penicillin procaine (Depocillin, Intervet, Salamanca, Spain). The second group was treated with 1 ml/10 kg body weight of a suspension of 150 mg/ml of amoxicillin trihydrate (Clamoxyl LA, Pfizer, Madrid, Spain). The third group was given 0.15 ml/kg body weight of 100 mg/ml of ampicillin (Ampilpen LA, Intervet). The products were administered according to the manufacturers’ instructions.

After treatment, the ewes were machine-milked twice a day. The milk yield from each ewe was measured, and samples were taken for analysis. Additional milk samples were collected every 12 h for up to 12 milking sessions. The withdrawal period recommended by manufacturers was five milkings for benzyl penicillin procaine and six milkings for amoxicillin and ampicillin. The samples (50 ml/milking session/animal) were collected in disposable plastic containers and kept at 4°C until they were analyzed.

Antibiotic detection tests. All milk samples were analyzed during the 24-h period after collection by simultaneously using four microbial growth inhibition assays—brilliant black reduction MRL test (BRT MRL), Copan test microplate P&S (CH ATK P&S), Delvotest SP, and Eclipse 100ov—and an enzymatic colormetric assay (Penzym 100). In all five tests, milk shown to be free from antimicrobials was used as “negative control.” This milk came from healthy animals that did not receive feed or treatments containing drugs. For the “positive control,” 4 µg benzyl penicillin G (Pen Na; SIGMA) per kg was added to negative milk samples. Visual interpretation was carried out independently by three trained observers and evaluated visually as “negative,” “doubtful,” or “positive.”

BRT MRL. One hundred microliters of milk was added to ready-prepared individual BRT MRL (AIM-Analytik in Milch Produktions- und Vertriebs GmbH, Munich, Germany) wells containing Bacillus stearothermophilus var. calidolactis. After a 1-h diffusion period at 4°C, the BRT MRL tests were incubated in a dry block heater at 64 ± 1°C for 2 h 30 min.

CH ATK P&S. After addition of 100 µl of milk samples into single wells of CH ATK P&S (Chr Hansen, Hoersholm, Denmark) containing spores of B. stearothermophilus var. calidolactis, plates were incubated at 64 ± 1°C for 3 h in a dry block heater.

Delvotest SP. One nutrient tablet and 100 µl of each milk sample were added to individual wells of Delvotest SP (DSM Food Specialties, Delft, The Netherlands), ready-prepared, containing B. stearothermophilus var. calidolactis. Plates were incubated in a dry block heater at 64 ± 1°C for 2 h 45 min.

Eclipse 100ov. Fifty microliters of each milk sample plus 50 µl of “ov 100” solution (ZEU-Immunotec, Zaragoza, Spain) were added to each well of Eclipse 100ov test (ZEU-Immunotec), ready-prepared, containing B. stearothermophilus var. calidolactis. Before being incubated at 64 ± 1°C for 2 h 30 min in a dry block heater, plates were preincubated 1 h at room temperature for milk and antibiotic diffusion.

Penzym 100. The Penzym 100 test (Chr Hansen) was carried out according to the manufacturer’s label inserts. The incubation period after addition of the chromogenic substrate was 22 min (27). Visual interpretation of the results by three trained persons was performed independently by comparison with a color table, as in the interpretation of microbial tests.

Statistical analysis. To evaluate the influence of the milking order and milk yield on the responses to the five screening tests studied, a logistic regression model (2) with the stepwise option from the logistic procedure of SAS was employed (32). Variables were analyzed using the following logistic model.
TABLE 2. Summary of the logistic regression model coefficients for penicillin depletion in ewe’s milk

<table>
<thead>
<tr>
<th>Assay</th>
<th>$\beta_0$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$C$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRT MRL</td>
<td>4.8063</td>
<td>7.5175</td>
<td>-1.1829</td>
<td>93.2</td>
</tr>
<tr>
<td>CH ATK P&amp;S</td>
<td>4.8550</td>
<td>6.0370</td>
<td>-1.1549</td>
<td>93.8</td>
</tr>
<tr>
<td>Delvotest SP</td>
<td>6.8687</td>
<td>9.3303</td>
<td>-1.7635</td>
<td>96.2</td>
</tr>
<tr>
<td>Eclipse 100ov</td>
<td>10.2911</td>
<td></td>
<td>-2.7418</td>
<td>98.5</td>
</tr>
<tr>
<td>Penzym 100</td>
<td>3.2016</td>
<td>4.7733</td>
<td>-0.9108</td>
<td>90.3</td>
</tr>
</tbody>
</table>

$^a \beta_0 = \beta_0$ was used to estimate the frequency of positive/doubtful + negative cases.

$^b \beta_2 = \beta_2$ was used to estimate the frequency of positive + doubtful/negative cases.

$^c C$, concordance coefficients.

\[
L_{ijk} = \logit[P_{ijk}] = \beta_0 + \beta_1[MO]_j + \beta_2[P]_j + \epsilon_{ijk}
\]

$L_{ijk}$ is the logit model, $[P_{ijk}]$ is the probability for the response category (positive/doubtful + negative or doubtful + positive/negative), $\beta_0$ is the intercept, $\beta_1$ and $\beta_2$ are the estimated parameters for the model, [MO] is the effect of milking order (1 through 7, and 12), $[P]_j$ is the effect of milk production, and $\epsilon_{ijk}$ is the residual error.

The concordance coefficient was applied as a rank correlation between the observed responses and predicted probabilities (32).

RESULTS AND DISCUSSION

Benzyl penicillin procaine treatment. Table 2 presents coefficients from the logistic regression model. For all assayed methods, the effect of milking order was significant ($P < 0.0001$) but not milk yield. Molina et al. (26) observed that milk production didn’t contribute significantly to persistence of penicillin in milk from ewes treated intramuscularly. Also, Seymour et al. (34) did not find significant effects for this factor in dairy cows. Nevertheless, sheep with lower milk production took longer to eliminate antibiotics other than penicillin supplied via other administration routes, such as intravenous or intramammary (26). Also, at the end of lactation, when milk production is low, a significant effect of milk yield, even by intramuscular route, has been found (5).

Higher values for $\beta_1$ coefficients showed by Eclipse 100ov (−2.7418; Table 2) and Delvotest SP tests (−1.7635; Table 2) revealed a steeper slope in the evolution of the positive results for these methods than did coefficients for the other methods (Fig. 1).

BRT MRL and Delvotest SP methods had elevated frequencies for doubtful tests in the period between the fourth and seventh milkings (Fig. 1). This result is associated with the $\beta_2 = \beta_2$ parameter from the logistic model because BRT MRL (7.5175 − 4.8063) and Delvotest SP (9.3303 − 6.6867) methods showed higher $\beta_2 = \beta_0$ values. For this antibiotic, no doubtful responses were provided with Eclipse 100ov.

At the first milking after the withdrawal period (five milkings), positive responses ranged from 2% (Delvotest SP) to 11% (CH ATK P&S), with only the Eclipse 100ov test providing all negative results (Fig. 1), perhaps because the first methods possess detection limits under the MRLs (between 1.4 and 3 μg/kg for Delvotest SP (7) and Penzym 100 (3), respectively). However, Eclipse 100ov has a detection limit of 5 μg/kg (6), over the MRLs for penicillin (4 μg/kg (10, 12)). Eclipse 100ov is a specific ewe’s milk test (14), whereas the other tests were developed for cow’s milk and subsequently evaluated to apply to ewe’s milk (3, 4, 7, 27). On the other hand, there could be a slight variation between the sensitivity when detecting residues in milk fortified “in vitro” and milk from treated animals, where natural inhibitors could also interfere. Moreover, small differences in the molecular structure of penicillin, as in penicillin G versus benzyl penicillin procaine, might provoke variations in method responses, as with Delvotest SP.

Ampicillin treatment. The results from the logistic model used to estimate the responses for the screening methods are summarized in Table 3. As in the penicillin treatment, only the milking order for the five tests was significant ($P < 0.0001$). For that reason, the effect of milking order on the frequency of positive and doubtful responses to the assayed methods is shown in Figure 2.

Eclipse 100ov (−3.3116), BRT MRL (−2.4580), and Delvotest SP (−2.0065) tests presented the highest $\beta_1$ coefficients. This reveals a faster decline of positive responses with these tests throughout the experimental period.

As with the benzyl penicillin procaine treatment, the $\beta_2 = \beta_2$ parameter was higher in BRT MRL (16.6004 − 10.0290) and Delvotest SP (11.2768 − 7.7846) tests, corresponding to the higher frequencies of doubtful tests observed in Figure 2 for BRT MRL and Delvotest SP. These high frequencies could be due to the coincidence of the
TABLE 3. Summary of the logistic regression model coefficients for ampicillin depletion in ewe’s milk

<table>
<thead>
<tr>
<th>Assay</th>
<th>$\beta_{01}\ a$</th>
<th>$\beta_{02}\ b$</th>
<th>$\beta_1$</th>
<th>C (%) $c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRT MRL</td>
<td>10.6290</td>
<td>16.6004</td>
<td>-2.4580</td>
<td>94.7</td>
</tr>
<tr>
<td>CH ATK P&amp;S</td>
<td>8.5030</td>
<td>10.0424</td>
<td>-1.7080</td>
<td>96.5</td>
</tr>
<tr>
<td>Delvotest SP</td>
<td>7.7846</td>
<td>11.2768</td>
<td>-2.0065</td>
<td>95.0</td>
</tr>
<tr>
<td>Eclipse 1000ov</td>
<td>13.6978</td>
<td>14.4512</td>
<td>-3.3116</td>
<td>98.4</td>
</tr>
<tr>
<td>Penzym 100</td>
<td>4.2916</td>
<td>5.7963</td>
<td>-0.9826</td>
<td>91.5</td>
</tr>
</tbody>
</table>

$^a\beta_{01} = \beta_0$ was used to estimate the frequency of positive/doubtful + negative cases.

$^b\beta_{02} = \beta_0$ was used to estimate the frequency of positive + doubtful/negative cases.

$^cC$, concordance coefficients.

detection limits of the assayed test with the MRLs at the end of the withdrawal time. On the other hand, some authors (22) observed a diminution in the frequency of doubtful cases when analyses were confirmed 24 h after heating to 82°C. For this reason, we recommend that laboratories and dairy industries repeat analyses of doubtful results after 24 h to define milk use and avoid the entrance of contaminated milk into the food chain.

The ampicillin manufacturer’s directions establish six milkings as the recommended withdrawal period. As Figure 2 indicates, there are almost no positive responses in the seventh milking, except for the Penzym 100 test, which showed 7%. Sensitivity to the tested methods is close to the ampicillin MRL (4 μg/kg (10, 12): within 3 μg/kg for Delvotest SP (7) and BRT MRL (15) and 4 μg/kg for Eclipse 1000ov (28), Penzym 100 (3), and CH ATK P&S (13).

Amoxicillin treatment. Statistical coefficients, obtained by means of the logistic model, to the response of the detection inhibitor methods in milk samples from amoxicillin-treated ewes are given in Table 4. As in previous treatments, Eclipse 1000ov ($-1.8235$) and Delvotest SP ($-1.2277$) tests showed the highest $\beta_1$ coefficients. In this case, the methods that showed higher doubtful responses were Eclipse 1000ov, Delvotest SP, and Penzym 100 (Table 4 and Fig. 3). High frequencies of positive responses were observed (73.4% CH ATK P&S, 51.7% BRT MRL, 28.4% Penzym 100, and 11.3% Delvotest SP) beyond the rec-

TABLE 4. Summary of the logistic regression model coefficients for amoxicillin depletion in ewe’s milk

<table>
<thead>
<tr>
<th>Assay</th>
<th>$\beta_{01}\ a$</th>
<th>$\beta_{02}\ b$</th>
<th>$\beta_1$</th>
<th>C (%) $c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRT MRL</td>
<td>6.8887</td>
<td>7.9490</td>
<td>-0.9742</td>
<td>92.3</td>
</tr>
<tr>
<td>CH ATK P&amp;S</td>
<td>6.9055</td>
<td>7.8484</td>
<td>-0.8418</td>
<td>90.1</td>
</tr>
<tr>
<td>Delvotest SP</td>
<td>6.5344</td>
<td>8.8514</td>
<td>-1.2277</td>
<td>94.2</td>
</tr>
<tr>
<td>Eclipse 1000ov</td>
<td>9.2268</td>
<td>11.6771</td>
<td>-1.8235</td>
<td>96.6</td>
</tr>
<tr>
<td>Penzym 100</td>
<td>3.9108</td>
<td>5.8315</td>
<td>-0.6905</td>
<td>86.7</td>
</tr>
</tbody>
</table>

$^a\beta_{01} = \beta_0$ was used to estimate the frequency of positive/doubtful + negative cases.

$^b\beta_{02} = \beta_0$ was used to estimate the frequency of positive + doubtful/negative cases.

$^cC$, concordance coefficients.

FIGURE 2. Relative frequency of positive (●) and doubtful (□) cases in ewe’s milk treated intramuscularly with ampicillin using BRT MRL, CH ATK P&S, Delvotest SP, Eclipse 1000ov, and Penzym 100 tests.

FIGURE 3. Relative frequency of positive (●) and doubtful (□) cases in ewe’s milk treated intramuscularly with amoxicillin using BRT MRL, CH ATK P&S, Delvotest SP, Eclipse 1000ov, and Penzym 100 tests.
ommended withdrawal period of six milkings (Fig. 3), although this frequency was low in the Eclipse 100ov test (2.8%).

These observations could be justified with the detection limit for each method. In fact, detection limits for the BRT MRL and CH ATK P&S tests are 2 to 3 μg/kg (15) and 3.5 μg/kg (13), respectively, which is why it is possible to detect residues after the withdrawal time. For Delvotest SP (7) and Penzym 100 (3), detection limits are 5 μg/kg, which is near the MRLs (4 μg/kg (10, 12)), and showed high frequencies of doubtful cases (45.1% for Delvotest SP and 44.6% for Penzym 100) at the end of the withdrawal time, as in the ampicillin treatment. By contrast, the Eclipse 100ov test has a detection limit value of 7 μg/kg (6), which explains the lowest positive frequencies after the safety period (2.8%). For amoxicillin, positive and doubtful tests have been detected after the withdrawal period even when using methods with detection limits over the MRLs. These findings imply that extension of the withdrawal time to more than six milkings is recommended in order to carry out the EU normative.

A word of warning to the dairy laboratories and industries might be advisable because, even though farmers follow the manufacturer’s label instructions, positive and doubtful results could be obtained in ewe’s milk from individual animals when samples are analyzed with BRT MRL, Delvotest SP, CH ATK P&S, and Penzym 100 tests, perhaps because the majority of the tests show detection limits under that of the MLR established for the antibiotics studied. Therefore, it is necessary to adapt these tests so that the detection limits are on par with the MRLs established.

However, it is necessary to take into account a different approach in the concept of “residue limit” because a position that considers the potential technological problems derived from residue persistence, and not just safety troubles, would be more desirable. According to Adriany (1), MRLs only represent safety toxicological levels; however, the microbiological effects in the dairy industry and the safety technological levels are not considered in this evaluation. For this reason, it is important to harmonize the analysis concept between industry and reference laboratories to avoid penalties for producers and to avoid technological problems.

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