Occurrence of Antibiotic Residues in Milk from Manchega Ewe Dairy Farms

M. Yamaki,1,2 M. I. Berruga,1,2 R. L. Althaus,3 M. P. Molina,4 and A. Molina1,2

1Departamento de Ciencia y Tecnología Agroforestal, ETSIA, and
2Sección de Calidad Alimentaria, Instituto de Desarrollo Regional,
Universidad de Castilla-La Mancha, 02071 Albacete, Spain
3Cátedra de Biofísica, Facultad de Ciencias Veterinarias,
Universidad Nacional del Litoral, 3080 Esperanza, Argentina
4Departamento de Ciencia Animal, Universidad Politécnica,
46071 Valencia, Spain

ABSTRACT

Ewe milk samples from different ovine dairy farms from the Castilla–La Mancha region of Spain were collected from bulk tanks to estimate the occurrence of antibiotic residues in raw and heated (82°C, 10 min) milk by the Delvotest SP test. The month of collection, somatic cell counts, and bacteriology were analyzed and examined by means of a logistic regression model. The screening of a total of 2686 raw milk samples showed 1.7% “positive” and 2.1% “doubtful” results, which decreased after heating treatment to 1.3% and 0.4%, respectively. “Positive” and “doubtful” samples were identified by penicillinase and p-aminobenzoic acid solutions, and the majority of them corresponded to antimicrobials different than β-lactams or sulfonamides. By applying a logistic regression model, a significant effect of month of collection and bacteriology was observed in the initial screening and after the heat treatment. The highest percentages of “positive plus doubtful” results were observed in late summer–early autumn. A slight peak was also observed in spring in raw milk samples. Bacteriology was positively correlated with “positive plus doubtful” results.

(Key words: ewe milk, antibiotic residue, screening test, somatic cell count)

Abbreviation key: PABA = p-aminobenzoic acid, PDO = Protected Denomination of Origin.

INTRODUCTION

As in cow milk, the presence of antimicrobial residues in ewe milk could cause serious health problems for consumers in the form of antibiotic resistance or allergies (EMEA, 1999), as well as for the dairy industry, in the form of delays in bacteriological processes used to manufacture dairy products (Mäyrä-Mäkinen, 1995).

These risks have led the European Union to establish the National Residue Monitoring Plan (Council Directive 96/23/CE) for substances and residues (Annex I) in certain animal products, including milk from nonbovine species, as well as guidelines for the level and frequency of sampling (Commission Decision 97/747/CE).

Castilla–La Mancha is the second largest region in Spain in ewe milk production (MAPYA, 2002a) and the largest producer of cheese under one official label of Protected Denomination of Origin (PDO) (MAPYA, 2002b). According to the aforementioned EU regulations, the occurrence of antibiotic residues in milk from this species must be studied because, in many instances, the production of dairy products starts with raw milk.

Microbial screening tests for antibiotic residues are currently used to detect antibiotic contamination in milk. One of them, based on the growth of Bacillus stearothermophilus var. calidolactis, is the Delvotest SP test, which has been successfully used for ewe milk (Althaus et al., 2002, 2003a; Molina et al., 2003a) and which is widely used for milk quality control in the official laboratories of the European Union.

The objective of this study was to investigate the occurrence of antimicrobial residues in ewe milk in the Castilla–La Mancha region (Spain) using the microbiological Delvotest SP test, by evaluating the main factors that influence the results, such as month of collection, SCC, and bacteriology.

MATERIALS AND METHODS

Milk Sample Collection and Analysis

Ewe milk samples from bulk tanks were collected monthly from 490 farms of Manchega ewe flocks that supplied milk for PDO Manchego cheese production. Samples were collected in 2 100-mL disposable plastic
Antibiotic Microbiological Screening Test  
Milk samples were analyzed before a 48-h postcollection period by the Delvotest SP test (DSM Food Specialties, Delft, The Netherlands). Antibiotic-free milk samples were used as “negative” controls. As “positive” controls, milk samples with 4 μg/kg benzyl penicillin G (Sigma Chemical Co., St. Louis, MO) were used. The method was carried out according to the manufacturer’s instructions and was incubated in a dry block heater at 64 ± 1°C for 2 h and 45 min. Visual interpretation was carried out by 3 qualified individuals and evaluated as “negative” (blue) or “positive” (yellow). “Doubtful” qualifications were considered “positive” (Suhren et al., 1996). For the statistical calculations, those visual results that presented at least 2 similar interpretations were considered. The detection limits of antibiotics in ewe milk by the Delvotest SP test have been reported by Althaus et al. (2002).

Heating Treatment  
“Positive” and “doubtful” samples were kept refrigerated at 4°C and 24 h later were heated to 82°C for 10 min (Molina et al., 2003b) for confirmation.

Identification of the Substances  
To identify the presence of β-lactam or sulfonamide compounds, “positive” and “doubtful” samples were tested after heat confirmation by the penicillinase and p-aminobenzoic acid (PABA) solutions. Four 100-μL aliquots of each sample were added to 4 wells of the Delvotest SP test, followed by, respectively, no addition, 10 μL of distilled water, 10 μL of penicillin solution (100,000 IU/mL; Cod. 9120, AiM-Analytik in Milch Produktions-und Vertriebs-GmbH, München, Germany), and 10 μL of PABA solution (3 mg/mL; Cod. A-9878, Sigma Chemical Co.). Table 1 lists the result of each series of 4 wells.

Table 1. Data interpretation for samples identified by penicillinase and p-aminobenzoic acid (PABA) solutions.

<table>
<thead>
<tr>
<th>100-μL sample</th>
<th>100-μL sample</th>
<th>100-μL sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>100-μL sample</td>
<td>100-μL sample</td>
<td>100-μL sample</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Interpretation</td>
<td>Interpretation</td>
</tr>
<tr>
<td>NIS²</td>
<td>β-Lactam</td>
<td>Sulfonamide</td>
</tr>
<tr>
<td>β-Lactam and sulfonamide</td>
<td>DS³</td>
<td></td>
</tr>
</tbody>
</table>

¹(+) = Positive or doubtful result; (-): negative result.
²NIS = No identified substance (antimicrobial substances different than β-lactams or sulfonamides).
³DS = Diluted samples.

Statistical Analysis  
Data were treated using a logistic regression model (Agresti, 1990). The “positive” and “doubtful” results by the Delvotest SP test were grouped as “positive plus doubtful” to treat these qualitative variables at two levels (“negative” and “positive plus doubtful”). The statistical design for analyzing the effects of month of collection, SCC, and bacteria upon the visual interpretation of the Delvotest SP test was carried out with the following logistic model:

\[ L_{ijklmn} = \beta_0 + \beta_1 T_1 + \beta_2 T_2^2 + \beta_3 T_3^3 + \beta_4 T_4 + \beta_5 \text{SCC}_m + \beta_6 \text{BAC}_n + \epsilon_{ijklmn} \]

where \( L_{ijklmn} \) is the variable logit, i.e., \( \ln \frac{P_{ijklmn}}{1 - P_{ijklmn}} \);
\( P_{ijklmn} \) is the probability of a “positive and doubtful” result; \( I - P_{ijklmn} \) is the probability of a “negative” result; \( \beta_0 \) to \( \beta_6 \) are coefficients estimated for the logistic regression models; \( T_1 \) is the effect of month squared; \( T_2^2 \) is the effect of month squared; \( T_3^3 \) is the effect of month cubed; \( T_4 \) is the effect of month quadrupled; SCC_m is the effect of somatic cells; BAC_n is the effect of bacteria; and \( \epsilon_{ijklmn} \) is the residual error.

The results were achieved using the STEPWISE option from the LOGISTIC procedure of SAS (SAS, 1998).

RESULTS AND DISCUSSION  
The initial screening of 2686 ewe milk samples by the Delvotest SP test showed 1.7% “positive” and 2.1%
Table 2. Summary of the logistic regression model coefficients for the initial analysis and the confirmation after heating at 82°C for 10 min by Delvotest SP test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter</th>
<th>Estimate</th>
<th>$\chi^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial analysis</td>
<td>Coefficient</td>
<td>-4.6596</td>
<td>24.9916</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Month</td>
<td>2.2830</td>
<td>6.2575</td>
<td>0.0124</td>
</tr>
<tr>
<td></td>
<td>Month(^2)</td>
<td>-0.6976</td>
<td>6.1325</td>
<td>0.0133</td>
</tr>
<tr>
<td></td>
<td>Month(^3)</td>
<td>0.0762</td>
<td>5.2532</td>
<td>0.0219</td>
</tr>
<tr>
<td></td>
<td>Month(^4)</td>
<td>-0.00280</td>
<td>4.5213</td>
<td>0.0335</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>-0.00042</td>
<td>6.2877</td>
<td>0.0122</td>
</tr>
<tr>
<td>Confirmation after heating</td>
<td>Coefficient</td>
<td>-2.9795</td>
<td>146.2402</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Month(^2)</td>
<td>-0.0151</td>
<td>10.8486</td>
<td>0.0010</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>-0.00088</td>
<td>5.3275</td>
<td>0.0210</td>
</tr>
</tbody>
</table>

“doubtful” results. A higher incidence (6% and 2%, respectively) was reported in the same region by using the BRT AiM test for ewe milk preserved by acidiol (Molina et al., 1999). According to these results, the quality of the ewe milk from this breed seemed to be higher than 5 yr previous. Superior results were observed in other Spanish regions and other EU countries with an important representation of dairy ewes. Thus, 11% “positive” results have been detected using the Eclipse 100ov test and even higher percentages using a multiplate assay (Esnal et al., 2002). Continanza et al. (2003) reported 14% “positive” results in raw milk from ewes from the province of Roma (Italy) by means of the Copan (CH ATK) test.

Screenings of dairy goats also showed superior positive levels (12.7%) in raw milk (Marco et al., 2001). The occurrence of “positive” results in milk from small ruminants was rather higher than that evidenced in cow milk (0.03 to 0.5%) from other EU countries (Harding, 1993; Suhren, 2002; Suhren and Reichmuth, 2003; Suhren and Walte, 2003) or the United States (Sargeant et al., 1998; Ruegg and Tabone, 2000; FDA/CFSAN, 2001; Van Schaik et al., 2002). Frequently, ewe and goat milks are characterized by an elevated concentration of natural inhibitors related to higher SCC that, on many occasions, is not due to infection-related factors (Bencini and Pulina, 1997; Paape et al., 2001). The interference that provokes these compounds on numerous microbial tests has been widely discussed (Carlsson and Bjorck, 1987; Carlsson et al., 1989; Schiffmann et al., 1992; Althaus et al., 2003b). In addition, controls for residue detection have been applied to cow milk for a long time (Booth and Harding, 1986; Harding, 1993; Heeschen and Suhren, 1996; Suhren and Reichmuth, 2003; Suhren and Walte, 2003). Nowadays, the incidence of residues in milk from small ruminants is gaining more attention, and the situation of dairy sheep and goats is expected to improve over the next few years, just as it did for cow milk through official control programs and integrated detection systems.

To evaluate the effects of month of collection, SCC, and bacteriology on test results, a logistic regression model was used by means of which the qualifications “positive” and “doubtful” were grouped (“positive plus doubtful”). In this model the variables of month of collection and bacteriology were significant (Table 2), with a concordance correlation coefficient of 67.3%. By applying this logistic regression model (Figure 1A) to estimate the probability of “positive plus doubtful” as the functions of month and bacteriology, the results indicated an important peak of maximal percentages in late summer–early autumn. On the other hand, a slight peak was observed in spring. This result could be related to the reproductive performance carried out in the flocks of this breed (Gallego et al., 1994). Although a higher percentage of lambings occurs in the spring, the low seasonality of this breed points to mat-

![Figure 1](https://example.com/figure1.png)

Figure 1. “Positive and doubtful” rate (%) in ewe milk samples from the Castilla–La Mancha region at the initial analysis (A) and at the confirmation analysis after 24 h (B) (heated at 82°C for 10 min) by Delvotest SP from July 2002 to June 2003. (●) Bacteriology of 1.5 × 10⁶ cfu/mL. (○) Bacteriology of 0.5 × 10⁶ cfu/mL.
ing in the spring, which coincides with lambings and milk production during the late summer–early autumn. At that time, a concurrence between late-lactation of in-season lambings and early-lactation of out-of-season lambings takes place and explains the peak observed during this period. In both stages of lactation, levels of SCC and natural inhibitors are more elevated (Fuertes et al., 1998; Althaus et al., 2001a; Paape et al., 2001) and, as previously mentioned, might interfere with test results (Carlsson and Bjorck, 1987; Carlsson et al., 1989; Schiffmann et al., 1992; Althaus et al., 2003b).

The high ambient temperature in summer probably also leads to a drop in milk volume yield (Gallego et al., 1994; Sevi et al., 2004). As reported previously, animals with lower milk production show a more prolonged withdrawal period after antibiotic therapy (Althaus et al., 2003b). This could be related to farms with poor sanitary and hygienic conditions that, on many occasions, make necessary the application of antibiotic therapies. Moreover, the Castilla–La Mancha region often averages high temperatures from late spring to late summer (Gallego et al., 1994), which could favor greater microbial growth (Sevi et al., 2004).

In addition, the probability of obtaining “positive plus doubtful” cases went up with the increase in the bacteriology levels (Figure 1A), which was more patent in the period corresponding to the maximum peak of responses. These results could be related to farms with poor sanitary and hygienic conditions that, on many occasions, make necessary the application of antibiotic therapies. Moreover, the Castilla–La Mancha region often averages high temperatures from late spring to late summer (Gallego et al., 1994), which could favor greater microbial growth (Sevi et al., 2004).

The geometric mean SCC for the milk samples was $1.1 \times 10^6$ cells/mL, and 65% of them showed counts between $0.5 \times 10^6$ and $1.5 \times 10^6$ cells/mL (geometric mean of $0.99 \times 10^6$ cells/mL). Moreover, no significant effect of the variable SCC was found by applying the logistic regression model, which is in contrast to other studies in which a high concentration of SCC was associated with false positive results (Carlsson and Bjorck, 1987; Carlsson et al., 1989; Schiffmann et al., 1992; Althaus et al., 2003b). This could be due to the low dispersion of the counts from the majority of the samples checked.

Samples that showed “positive” or “doubtful” results were confirmed for the presence of inhibitors after heat treatment at 82°C for 10 min. Following this treatment, 1.3 and 0.4% of the total initial samples remained “positive” and “doubtful,” respectively. Several studies have pointed out the effect of naturally occurring inhibitors in cow milk under the growth of Bacillus stearothermophillus var. calidolactis, creating false positive results (Carlsson and Bjorck, 1987; Carlsson et al., 1989; Schiffmann et al., 1992). As the heating treatment has been shown to decrease the enzymatic activity of these natural inhibitors (Molina et al., 2003b), which are more active in ewe milk than cow milk (Althaus et al., 2001a), the reduction of 2.1% of the total “positive plus doubtful” results may be explained as false positives due to natural inhibitors. Althaus et al. (2003b) also found a decrease in false positives after heating ewe milk. This reduction might also be related to the presence of some thermosensitive antimicrobial compounds (Sanz et al., 2002; Zorraquino et al., 2002). The possibility of chlorine contamination of milk as a result of milking equipment sanitation is also connected with false positive responses (Contreras et al., 1997).

The logistic regression model for confirmation after heating is shown in Table 2 and Figure 1B, with a concordant correlation coefficient of 69.4%. After heat treatment, a reduction of “positive plus doubtful” results was observed that was greater in samples corresponding to the late summer–early autumn peak. This diminution might confirm a higher presence of indigenous inhibitors during this time.

The 47 samples detected as “positive plus doubtful” after heat treatment were identified by the penicillinase and PABA solutions (Table 3). Almost half of the samples (20) were not identified as β-lactams or sulfonamides, and 14 samples were identified as β-lactams. Neither sulfonamides nor combinations of sulfonamides and β-lactams were detected, results similar to those reported by Molina et al. (1999). The “positive plus doubtful” cases not identified by penicillinase or PABA could also be associated with nonantibiotic compounds that resist the heating treatment and have antimicrobial activity. A microbiological multiplate test used to analyze ewe milk indicated β-lactams, tetracyclines, and/or macrolides (Esnal et al., 2002).

On the other hand, a high number of samples could not be identified because of a dilution effect (samples diluted) within the penicillinase and PABA methodology. It is possible that these samples had antibiotic concentrations near to the detection limits of each antibiotic for the Delvotest SP. It has been reported that antibiotics in ewes could prolong the withdrawal period, which might explain these cases (Berruga et al., 2003; Molina et al., 2003a).

### Table 3. Identification of “positive and doubtful” samples (absolute and relative frequencies) after the p-aminobenzoic acid and penicillinase tests.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absolute frequency</th>
<th>Relative frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unidentified substance</td>
<td>20</td>
<td>42.6</td>
</tr>
<tr>
<td>β-Lactam</td>
<td>14</td>
<td>28.8</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β-Lactam and sulfonamide</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diluted samples</td>
<td>13</td>
<td>27.7</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>100</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The levels of “positive” and “doubtful” results observed by Delvotest SP in ewe milk from the Castilla–La Mancha region were 1.3% and 0.4%, respectively. That a reduction in “positive” and “doubtful” responses was observed after a heating treatment suggests that such a treatment should be performed on ewe milk before carrying out the microbial test thus avoiding incorrect interpretations. This decrease could indicate an important effect of natural inhibitors from ewe milk in the Delvotest SP test.

On the other hand, the milk samples from Castilla–La Mancha ewes presented a greater probability of “positive” or “doubtful” responses in late summer–early autumn, perhaps suggesting that more attention be given at this time to support milk quality throughout the year. The great influence of the bacteriology level on the results of this test were noted.

Finally, a higher proportion of samples confirmed after heating were not identified as β-lactams or sulfonamides by penicillinase and PABA solutions. According to these results, we can conclude that penicillinase and PABA solutions inadequately identify most of the “positive plus doubtful” compounds detected by microbial tests of ewe milk, and therefore further identification assays are necessary to establish the nature of these compounds.

ACKNOWLEDGMENTS

This work was funded by the project 191/IA-40 from the Consejería de Agricultura y Medio Ambiente (Junta de Comunidades de Castilla-La Mancha, Spain). The authors would like to thank the workers from the For-lactaria Operadores Lecheros S.A. dairy company (Vil-larrobledo, Albacete, Spain) for their valuable help in the collection of samples. The authors are also grateful to DSM Food Specialties (Delft, The Netherlands) for its support.

REFERENCES


Mäyrä-Mäkinen, A. 1995. Technological significance of residues for the dairy industry. Pages 136–143 in Symp. on Residues of Antimicrobial Drugs and Other Inhibitors in Milk. IDF Special Issue no. 95 05. Kiel, Germany.
