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Performance of Blue-Yellow Screening Test for Antimicrobial Detection in Ovine Milk

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ABSTRACT

Drug residues in milk are important because of public health and industrial implications. The detection limits of 25 antimicrobial agents were determined by the blue-yellow screening method in ovine milk. For each drug, 8 concentrations were tested on 20 ovine milk samples from individual ewes in midlactation. Detection limits determined by means of logistic regression were below European Union maximum residue limits (EU-MRL) for penicillin G (3 to 4 μg/kg), ceftiofur (96 to 107 μg/kg), framycetin (720 to 781 μg/kg), neomycin (915 to 1,084 μg/kg), and tylosin (44 to 51 μg/kg). Detection limits for ampicillin (5 to 6 μg/kg), cloxacillin (33 to 42 μg/kg), cefoperazone (73 to 82 μg/kg), cefalexin (160 to 202 μg/kg), gentamycin (355 to 382 μg/kg), streptomycin (3,063 to 3,593 μg/kg), tilmicosin (109 to 131 μg/kg), erythromycin (444 to 522 μg/kg), spyramicin (1,106 to 1,346 μg/kg), sulfadimethoxine (101 to 119 μg/kg), sulfathiazole (122 to 151 μg/kg), sulfamethazine (309 to 328 μg/kg), sulfanilamide (1,750 to 2,674 μg/kg), tetracycline (233 to 257 μg/kg), oxytetracycline (398 to 501 μg/kg), doxycycline (323 to 419 μg/kg), chlortetracycline (3,331 to 3,989 μg/kg), danofloxacin (4.7 to 5.5 mg/kg), enrofloxacin (41 to 46 mg/kg), and flumequin (63 to 71 mg/kg) were higher than the EU-MRL. Although the blue-yellow method showed improved sensitivity compared with other tests studied in ovine milk, the performance of screening methods for detecting antimicrobial agents in milk of this species should be improved.
Key words: ovine milk, screening test, detection limit, antimicrobial residue

INTRODUCTION

Ewe milk is used mainly in the production of fermented dairy products, especially cheese. The presence of antimicrobial residues (**AR**) in milk constitutes a potential hazard for the consumer because of allergic reactions, intestinal dysbiosis, and resistant populations of bacteria in the general population (Allison, 1985; Dewdney et al., 1991). In addition, AR in milk could cause serious technical problems for the dairy industry by inhibiting the bacterial processes involved in the elaboration of cheese and cultured milk products (Mourot and Loussouarn, 1981).

The European Union (**EU**) determines the limits for the presence of specified veterinary residues in milk. The antimicrobial residues are defined by Council Regulation EEC 2377/90 (EU, 1990), although a number of amendments have subsequently been made to extend the list of agents with maximum residue limits (**MRL**) established.

Increasing awareness of public health and food safety issues in recent years has lead to a greater interest in milk quality. To determine the presence of AR in cow milk, several rapid screening tests have been developed to test milk on the farm or in milk plants (IDF, 1991); recently, interest in research into AR detection is growing in dairy sheep (Berruga et al., 2003; Yamaki et al., 2004). As intensification of milk production in small ruminants has increased in recent years, the use of antimicrobial substances in dairy ewes has become a usual practice in veterinary medicine to treat mastitis and other diseases. In addition, the shortage of specific commercial formulations for dairy ewes makes it necessary to use antibiotic preparations normally used in cattle, the withdrawal period of which is undefined in ewe's milk. Within an AR testing program in this species, a broad study on AR detection methods is needed to guarantee residue levels in milk below the established EU-MRL. Validation of tests is essential for selection of the most appropriate testing strategies, estimation of predictive values, appropriate test interpretation, and to ensure that testing programs operate as efficiently as possible (Gardner, 1997). The first results obtained from the BRT-AiM (AIM-Analytik in Milch Producktions-und Vertriebs GmbH, Munich, Germany; Althaus et al., 2001; Molina et al., 2003), Delvotest-SP

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(DSM Food Specialities, Delft, the Netherlands; Althaus et al., 2003), and Eclipse-100ov (ZEU-Immunotec, Zaragoza, Spain; Montero, 2004) tests in detecting AR in ovine milk demonstrated low sensitivity and high variability of those methods, particularly for no-β-lactam AR.

The blue-yellow method (**BY**) is a broad-spectrum microbial inhibition assay for cow milk AR detection, and it is not well known in countries of the European Union. This simple and easy-to-read screening test gives results within a relatively short period (<3 h). Results of the BY test are classified visually into 3 categories: "negative," "doubtful," and "positive" compared with reference colors. According to manufacturer's instructions, the detection limits (**DL**) of this test for some macrolides, aminoglycosides, tetracyclines, and sulfonamides are close to MRL, and an attempt should be made to evaluate the test's performance in ovine milk.

Thus, the evaluation of this method for ovine milk could be of interest in programs for AR detection, and this study should increase the information about the performance of AR detection methods in dairy sheep. The aim of this research was to calculate the BY DL for 25 antimicrobial agents belonging to 6 different families in ovine milk.

MATERIALS AND METHODS

Individual ewe milk samples (50 mL) were collected in midlactation from Assaf ewes of the experimental flock located on the farm of the Department of Animal Production, University of León (Spain). The total flock size was 250 lactating ewes, and the selection of ewes for sampling was done randomly. The animals received no pharmacological treatment before the study, and samples corresponded to the morning machine milking (0800 h). Ewes with atrophic half-udders were excluded from this study. The flock was kept permanently in stalls, and they remained under similar environmental, handling, and feeding conditions. The SCC of bulk tank milk was always $\leq 500 \times 10^3$ cells/mL.

Milk samples were analyzed during the 4-h period after collection by BY test (Charm Sciences Inc., Lawrence, MA), which is a microbial growth inhibition assay intended for use on bulk tank milk and individual animal samples. After the addition of 50 μL of milk into single wells containing spores of *Geobacillus stearothermophilus* var. *calidolactis* ATCC 10149 strain, plates were incubated at 65°C for 2 h 45 min. Visual interpretation of results was carried out by comparison with a color table and evaluated as negative, doubtful, or positive.

In accordance with the IDF indications (IDF, 1999), 8 concentrations were prepared for each drug in the proximity of the test detection level. A previous study using dilutions (1:10) between 100 mg/kg and 0.1 μg/kg was carried out as a first approximation to DL for each antimicrobial agent. For each concentration, 20 replicates were prepared using 20 different antibiotic-free milk samples obtained from individual animals. The number of different individual milk samples was 125 (each sample was used to test the 8 concentrations of each drug in 4 different drugs). Samples were collected on the day of testing. The number of antimicrobial agents studied was 25, from 6 antimicrobial families. The list of drugs included 3 penicillins (penicillin G, ampicillin, cloxacillin), 3 cephalosporins (cefoperazone, cephalexin, and ceftiofur), 4 aminoglycosides (gentamycin, neomycin, framycetin, and streptomycin), 4 macrolides (tylosin, tilmicosin, spyramicin, and erythromycin), 4 tetracyclines (tetracycline, doxicycline, oxytetracycline, and chlortetracycline), 4 sulfonamides (sulfadimethoxine, sulfathiazole, sulfamethazine, sulfanilamide), and 3 quinolones (enrofloxacine, flumequine, and danofloxacine). Table 1 summarizes the antimicrobial agents and the concentrations used. These drugs were stored and handled according to the manufacturers' instructions before being used. Drugs were dissolved (1 mg/mL) in water, except ceftiofur (dissolved in Tris-HCl, 100 m*M*, pH 9); sulfanilamide, tetracycline, chlortetracycline, and oxytetracycline (dissolved in methanol); and erythromycin (dissolved in ethanol). The pH were adjusted with KOH or HCl. Final concentrations in milk (μg/kg) were achieved after serial dilutions in such a way that the volume of the antimicrobial agent solution did not exceed 1% of the volume of the final solution to be analyzed. In this study, the total number of observations was 4,000 (25 drugs × 8 concentrations × 20 replicates).

Because BY is a method with visual interpretation, reproducibility between observers was studied in 6 antimicrobial drugs, 1 from each antimicrobial family. The 8 concentrations of each antimicrobial agent were tested in 4 different ovine milks, and visual interpretation of results was carried out independently by 3 observers by comparison with a color table. Results were always evaluated as negative, doubtful, or positive. In this study, the total number of different observations was 192 (6 drugs × 8 concentrations × 4 milks), which were read by 3 independent observers.

Statistical Analyses

The DL of antimicrobial agents were estimated by a logistic regression model using the LOGISTIC procedure of SAS (SAS Institute, 1998). For this model, the

Table 1. Antimicrobial agents and concentrations used for blue-yellow detection limits in ovine milk

Antimicrobial class/agent	Product number[1]	Concentrations tested (μg/kg or *mg/kg)
β-lactams		
Penicillin G	Sigma Pen-Na	0.5, 1, 2, 3, 4, 5, 6, 7
Ampicillin	Fluka 10045	1, 2, 3, 4, 5, 6, 7, 8
Cloxacillin	Fluka 27555	5, 10, 20, 30, 40, 50, 60, 70
Cefalexine	Fluka 22238	25, 50, 100, 150, 200, 250, 300, 350
Ceftiofur	Riedel de Haën 34001	60, 70, 80, 90, 100, 110, 120, 130
Cefoperazone	Fluka 22129	40, 50, 60, 70, 80, 90, 100, 110
Aminoglycosides		
Gentamycin	Sigma G-3632	200, 250, 300, 350, 400, 450, 500, 550
Neomycin	Fluka 72133	600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300
Framycetin	Riedel de Haën 33492	300, 400, 500, 600, 700, 800, 900, 1,000
Streptomycin	Fluka 85880	1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5*
Macrolides		
Tylosin	Sigma T-6134	10, 20, 30, 40, 50, 60, 70, 80
Tilmicosin	Riedel de Haën 33864	60, 70, 80, 90, 100, 150, 200, 250
Erythromycin	Fluka 45673	100, 200, 300, 400, 500, 600, 700, 800
Spyramicin	Sigma S-9132	0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2*
Tetracyclines		
Tetracycline	Sigma T-3258	50, 100, 150, 200, 250, 300, 350, 400
Oxytetracycline	Fluka 75966	90, 100, 200, 300, 400, 500, 600, 700
Doxycycline	Fluka 44577	50, 100, 200, 300, 400, 500, 600, 700
Chlortetracycline	Sigma C-4881	0.5, 1, 1.5, 2, 3, 4, 5, 6*
Sulfonamides		
Sulfadimethoxine	Sigma S-7358	60, 70, 80, 90, 100, 200, 250, 300
Sulfathiazole	Sigma S-0127	80, 90, 100, 110, 120, 150, 200, 220
Sulfamethazine	Sigma S-5637	50, 100, 200, 300, 400, 500, 600, 700
Sulfanilamide	Sigma S-9251	0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5*
Quinolones		
Danofloxacine	Riedel de Haën 33700	1, 2, 3, 4, 5, 6, 7, 8*
Enrofloxacine	Fluka 17849	5, 10, 20, 30, 40, 50, 60, 70*
Flumequine	Riedel de Haën 45735	20, 30, 40, 50, 60, 70, 80, 90*

[1]Products obtained from Sigma (St. Louis, MO), Fluka (Buchs, Switzerland), and Riedel de Haën (Seelze, Germany).

response was considered as ordinal with 3 possible values, which corresponded to positive, doubtful, and negative results. The logistic regression model used was

$$L_{ij} = \text{logit}\,[P_{ij}] = a_0 + b\,AC_i + \varepsilon_{ij}$$

where logit = lineal logistic model; i.e., $ln\,[P_{ij}/(1 - P_{ij})]$; P_{ij} = probability of positive vs. doubtful + negative results, on the one hand, and positive + doubtful vs. negative results, on the other hand; AC = antimicrobial concentration; a = intercept; b = slope; and ε_{ij} = residual error. In this study, 2 intercept coefficients were obtained: a_{01}, for the estimation of frequency of positive vs. doubtful + negative results, and a_{02}, for the estimation of frequency of positive + doubtful vs. negative results. The concordance coefficients were also estimated. This coefficient was applied as rank correlation between observed and predicted results (Althaus et al., 2003). This model included all possible categorical results and provided 2 DL for each antimicrobial agent. The DL of visual interpretation for BY test was estimated as the concentration at which 95% of results were positive. Sensitivity was defined as the antimicrobial concentration that was detected by BY test.

According to Ortega et al. (1995), reproducibility between observers was evaluated by means of kappa value defined as (OP – EP)/(1 – EP), where OP = observed concordance between observers, and EP = random predicted concordance.

RESULTS AND DISCUSSION

Concordance of the BY test between observers was very high. Kappa values were 0.99, 0.96, and 0.96 between observers 1 and 2, 1 and 3, and 2 and 3, respectively, when doubtful results were considered as negative; and 0.97, 0.94, and 0.94 when doubtful results were considered as positive.

Table 2 summarizes the statistical and DL values. Two DL values were found for each antimicrobial agent: DL_1 for comparison between positive vs. doubtful + negative results, and DL_2 for comparison between positive + doubtful vs. negative results. The concordance percentages of logistic regression were high (85.7 to 97.7%;

Table 2. Summary of logistic regression model and blue-yellow detection limits (DL,[1] μg/kg) of 25 antimicrobials agents studied in ovine milk considering an ordinal response variable

Antimicrobial class/agent	Intercept 1 (a_{01})	Intercept 2 (a_{02})	Slope (b)	Concordance % (c)	DL_1	DL_2	MRL[2] (μg/kg)
β-Lactams							
Penicillin G	–17.718	–14.468	4.608	97.3	4	3	4
Ampicilin	–14.480	–12.088	3.035	96.3	6	5	4
Cloxacillin	–49.295	–38.358	1.232	94.5	42	33	30
Cefoperazone	–98.069	–87.197	1.226	94.5	82	73	50
Ceftiofur	–44.633	–39.629	0.442	97.1	107	96	100
Cefalexin	–49.024	–38.215	0.256	89.1	202	160	100
Aminoglycosides							
Gentamycin	–138.600	–128.600	0.370	87.4	382	355	100
Framycetin	–27.233	–24.883	0.039	97.3	781	720	1,500
Neomycin	–49.557	–44.293	0.048	97.7	1,084	915	1,500
Streptomycin	–86.183	–73.015	0.025	85.7	3,593	3,063	200
Macrolides							
Tylosin	–13.888	–11.847	0.332	96.8	51	44	50
Tilmicosin	–18.263	–14.659	0.162	96.3	131	109	50
Erythromycin	–66.479	–56.120	0.133	97.5	522	444	40
Spyramycin	–12.405	–9.670	0.011	96.0	1,346	1,106	200
Tetracyclines							
Tetracycline	–92.642	–83.533	0.371	98.6	257	233	100
Oxytetracycline	–14.351	–10.796	0.034	96.8	501	398	100
Doxycycline	–47.667	–36.014	0.121	88.8	419	323	—
Chlortetracycline	–11.262	–8.918	0.004	96.9	3,989	3,331	100
Sulfonamides							
Sulfadimethoxine	–22.838	–18.948	0.215	95.9	119	101	100
Sulfathiazole	–30.556	–23.999	0.221	88.3	151	122	100
Sulfamethazine	–38.625	–36.271	0.127	98.3	328	309	100
Sulfanilamide	–13.587	–7.879	0.006	94.5	2,674	1,750	100
Quinolones							
Danofloxacine	–18.765	–15.955	0.003	97.1	5,495	4,783	30
Enrofloxacine	–83.582	–73.795	0.002	84.1	46[3]	41[3]	100
Flumequine	–12.333	–10.535	0.0002	94.4	71[3]	63[3]	50

[1]DL_1 = detection limit for positive vs. doubtful + negative; DL_2 = detection limit for positive + doubtful vs. negative.

[2]MRL = European Union maximum residue limits.

[3]Values in mg/kg.

Table 2) illustrating the good correlation achieved between observed and predicted results by logistic regression.

The doubtful results should only be considered as positive if the DL of an antimicrobial drug was greater than the MRL, but if the DL was smaller than MRL, then the doubtful results were negative. In addition, the concordance between observers was very high. Clear positive or negative results were easily identified by 3 observers, but some discrepancy between observers was possible for concentrations close to DL. This discrepancy was considered by the model of logistic regression used in the statistical study, in which doubtful results were grouped with positive or negative results. So, 2 DL were obtained showing a sensitivity interval for each antimicrobial agent for any observer. So, the logistic regression considering 2 DL seemed more appropriate than logistic regression based in binary response with 1 DL only (i.e., positive + doubtful vs. negative results) used in other studies (Althaus et al., 2001, 2003).

The coefficient b (slope) of logistic regression is a parameter closely related to the screening test sensitivity for each antimicrobial agent. A smaller b coefficient produced greater DL values and consequently less BY sensitivity for any antimicrobial agent. The lowest b values were for quinolones (0.0002 to 0.003) and the greatest values were for penicillin G (4.6) and ampicillin (3.03; Table 2).

The b parameter reached greater values for β-lactams than for the other chemotherapeutics assayed. The DL calculated for penicillin G (3 to 4 μg/kg) and ceftiofur (96 to 107 μg/kg) were similar to or below EU-MRL (4 and 100 μg/kg, respectively). The DL for ampicillin (5 to 6 μg/kg), cloxacillin (33 to 42 g/kg), cefoperazone (73 to 82 μg/kg), and cefalexin (160 to 202 μg/kg) were greater than EU-MRL (4, 30, 50, and 100 μg/kg). These DL were very similar to found by other authors using

the BRT, Eclipse-100ov or Delvotest-SP screening tests in ovine milk (Althaus et al., 2001, 2003; Montero, 2004), although the DL for penicillin (1 μg/kg) and cephalexin (40 μg/kg) were lower in the Delvotest-SP test (Althaus et al., 2003). β-Lactams can be effective against gram-positive pathogens and they are frequently used in mastitis therapies for dairy sheep (Marco, 1994; Molina et al., 2003, Linage et al., 2007), so a detection program for β-lactams in milk has been implemented in the main dairy sheep basins (i.e., Eclipse 100ov method in Castilla y León, Spain).

Framycetin (720 to 781 μg/kg) and neomycin (915 to 1,084 μg/kg) had DL lower than those found by using the Delvotest-SP (2,600 μg/kg), BRT-AiM (3,700 μg/kg), and Eclipse-100ov (9,100 μg/kg) tests for neomycin in ovine milk (Althaus et al., 2003; Molina et al., 2003; Montero, 2004). Results for framycetin using other tests than BY are unknown in ovine milk. In addition, the DL for gentamycin (355 to 382 μg/kg) and streptomycin (3,063 to 3,593 μg/kg) were also lower than those obtained by using the Delvotest-SP, BRT-AiM, or Eclipse-100ov tests (1,200 to 1,950 μg/kg for gentamycin, and 6,100 to 10,000 μg/kg for streptomycin; Althaus et al., 2003; Molina et al., 2003; Montero, 2004). Consequently, BY had greater sensitivity than other screening tests for detecting aminoglycosides. Nevertheless, only DL for neomycin and framycetin obtained by BY in ovine milk were lower than EU-MRL (1,500 μg/kg). This screening test is not appropriate, however, for gentamycin (EU-MRL: 100 μg/kg) or streptomycin (EU-MRL: 200 μg/kg), which showed the lowest b values (0.37 and 0.03). Neomycin and framycetin are antimicrobial agents used in mastitis treatments (i.e., ewe dry therapies) because of their effectiveness against gram-negative organisms, so the high sensitivity showed for BY in detecting these drugs must be emphasized.

Within the macrolides, only tylosin (44 to 51 μg/kg) showed a DL very close to EU-MRL (50 μg/kg), but the DL for tilmicosin (109 to 131 μg/kg), erythromycin (444 to 522 μg/kg), and spyramicin (1,106 to 1,346 μg/kg) were greater than EU-MRL (50, 40, and 200 μg/kg, respectively). Althaus et al. (2003) and Montero (2004) reported greater DL for tylosin (100 to 220 μg/kg), erythromycin (700 to 980 μg/kg), and spyramicin (15,500 μg/kg) when using the Eclipse-100ov and Delvotest-SP tests in ovine milk. As a result, BY showed a great sensitivity for macrolides compared with the abovementioned screening tests, but only tylosin could be detected at concentrations below or at the EU-MRL. Tylosin is an antibiotic developed for veterinary use with a variable activity again gram-positive and mycoplasma organisms. It is frequently used for contagious agalactia treatment in enzootic areas in case of clinical outbreaks (i.e., Mediterranean countries), and consequently, detection programs based on an appropriate screening test should be established.

Results obtained by using BY for tetracyclines demonstrated that this test was more sensitive for tetracycline with a DL (233 to 257 μg/kg) greater than EU-MRL (100 μg/kg) but lower than DL obtained by using Eclipse-100ov (480 μg/kg), Delvotest-SP (590 μg/kg), or BRT-AiM (6,200 μg/kg; Althaus et al., 2003; Montero, 2004; Molina et al., 2003). The BY test had a sensitivity similar to other methods for doxycycline (323 to 419 μg/kg), a very improved sensitivity for oxytetracycline (398 to 501 μg/kg) compared with the BRT-AiM test (5,500 μg/kg; Molina et al., 2003), and a low sensitivity for chlortetracycline (3,331 to 3,989 μg/kg). Nevertheless, the tetracycline family, and particularly chlortetracycline, showed DL clearly separate from EU-MRL (100 μg/kg).

The DL for sulfadimethoxine (101 to 119 μg/kg) was slightly greater than EU-MRL (100 μg/kg). Sulfathiazole (122 to 151 μg/kg), sulfamethazine (309 to 328 μg/kg), and particularly sulfanilamide (1,750 to 2,674 μg/kg) had DL greater than EU-MRL (100 μg/kg). The sulfonamides were better detected by BY than by other screening tests such as Eclipse-100ov (170 to 750 μg/kg), except for sulfanilamide, which was better detected by the Eclipse-100ov test (370 μg/kg; Montero, 2004). The BRT-AiM test DL for the sulfonamide family (3,200 to 6,500 μg/kg; Molina et al., 2003) were much greater than those obtained by the BY test.

The DL for quinolones (4.7 to 71 mg/kg; Table 2) were much greater than EU-MRL (30 to 100 μg/kg) and similar to results reported for the Eclipse-100ov test (3.7 to 90 mg/kg; Montero, 2004).

Comparing our results with those obtained by using other screening tests, it must be emphasized that there are important differences among methods for antimicrobial DL, particularly for several aminoglycosides, macrolides, and sulfonamides, despite the fact that all these methods use microbial inhibitor procedures based on inhibition of spore outgrowth of *G. stearothermophilus* var. *calidolactis*. In this sense, different performances among methods cannot be fully explained by differences in strain types used in each test (i.e., BY and Eclipse tests are based on the same strain of *G. stearothermophilus*: ATCC 10149). Thus, the concentration of organisms within individual wells and the properties of the gel or culture medium in which the organisms are placed could be important in increasing the sensitivity of the screening test, but this information is not available.

This study was carried out in individual Assaf milk samples and in midlactation. Early lactation is also an important period for an increased risk of antibiotic residues, but some screening tests can show high rates

of false-positive outcomes in colostrum. A previous study using BY to evaluate the residue status in colostrum demonstrated a BY specificity rate of 0.966 in Assaf ewes (Linage et al., 2007). This is an increased rate compared with other screening tests used in dairy cattle (Andrew, 2001), so BY could be used in early and midlaction in dairy sheep.

CONCLUSIONS

For antimicrobial drugs whose DL were similar to those established as EU-MRL, the following values, calculated by means logistic regression, were obtained by BY: 3 to 4 μg/kg for penicillin G; 96 to 107 μg/kg for ceftiofur; 720 to 781 μg/kg for framycetin; 915 to 1,084 μg/kg for neomycin; and 44 to 51 μg/kg for tylosin. In contrast, sensitivity was low or very low for the remainder of antimicrobial agents studied, although BY showed improved sensitivity compared with other screening tests studied in ovine milk. For this reason, we would recommend improvement in the sensitivity of screening tests to detect a greater number of residues of antimicrobial agents in ovine milk.

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