Romero Rueda, T.; Beltrán Martínez, MC.; Lisandro Althaus, R.; Molina Pons, MP. (2014). Detection of antibiotics in goat's milk; effect of detergents on the response of microbial inhibitor tests. Journal of Dairy Research. 81(3):372-377. doi:10.1017/S0022029914000259.

Detection of antibiotics in goat's milk: effect of detergents on the response of microbial inhibitor tests

T. Romero [a], M.C. Beltrán[a], R.L. Althaus [b], M.P. Molina[a]

[a]*Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València, Camino de Vera S/N, 46022. Valencia, Spain.*

[b]*Cátedra de Biofísica. Facultad de Ciencias Veterinarias. Universidad Nacional del Litoral. R.P.L., Kreder. 3080. Esperanza, Argentina.*

Corresponding author: Tamara Romero Rueda

Address: Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain

Telephone number: +34 96 387 43 25

Fax number: +34 96 387 74 39

e-mail address: tarorue@upvnet.upv.es

Summary

The aim of the study was to evaluate the interference of acid and alkaline detergents employed in the cleaning of milking equipment of caprine dairy farms on the performance of microbial tests used in antibiotic control (BRT MRL, Delvotest MCS, and Eclipse 100). Eight concentrations of commercial detergents, five acid (0-0.25 %) and five alkaline (0-1 %) were add to antimicrobial-free goat's milk to evaluate the detergent effect on the response of microbial inhibitor tests. To evaluate the effect of detergents on the detection capability of microbial tests two detergents at 0.5 ml/l (an acid one and a basic one) and eight concentrations of four β-lactam antibiotics (ampicillin, amoxicillin, cloxacillin and penicillin G) were used. Milk without detergents was used as control. The spiked samples were analysed twelve times by three microbial tests. The results showed that the presence of acid detergents did not affect the response of microbial tests for any of the concentrations tested. However, at concentrations equal or greater to 2 ml/l of alkaline detergents positive results were found in microbial tests (16.7-100%). The detection limits of the screening tests for penicillins were not modified substantially by the presence of detergents. In general, the presence of acid and alkaline detergents in goat's milk did not produce a great interference in the microbial tests, only high concentrations of detergents could cause non-compliant results, but these concentrations are difficult to find in practice if proper cleaning procedures are applied in goat dairy farms.

Keywords: detergents, inhibitors, screening methods, goat's milk

Introduction

Veterinary drug residues in milk are a growing concern among consumers, because of the risk they might pose for health, i.e. generating allergies, toxic reactions or drug resistance (Alanis, 2005; Demoly & Romano, 2005; Sanders *et al*., 2011), and technological implications in the

manufacture of dairy products (Packham *et al*., 2001; Adetunji, 2011). Therefore, Maximum Residue Limits (MRLs) of drugs in different foodstuffs of animal origin, including milk, have been defined by Regulation (EC) 470/2009 (European Union, 2009) and established by Commission Regulation (EU) 37/2010 (European Union, 2010).

Currently, there are numerous screening tests commercially available to detect antimicrobial residues in milk (IDF, 2010). In control laboratories, microbial inhibitor tests are widely used thanks to their simplicity, low cost and wide range of detection. Microbial inhibitor tests are based on the inhibition of spore outgrowth of the microorganism-test, the most commonly applied being *Geobacillus stearothermophilus* var. *calidolactis*; a thermophilic bacterium highly sensitive to β-lactam antibiotics. Screening microbial tests are non-specific methods and may be affected by different substances capable of inhibiting microorganism-test growth, causing positive results in antibiotic-free milk samples, such us: natural inhibitors of milk (Andrew, 2001), and preservatives (Molina *et al*., 2003), among others.

Detergents and disinfectants used in the cleaning of milking parlours and milk tanks are a possible source of residues in milk and have occasionally been associated with the positive results obtained in microbial tests (Fabre *et al*., 1995).

The hygienic production of milk implies the use of cleaning products to prevent the proliferation of microorganisms on surfaces that come into direct contact with milk, such as milking machines and milk storage tanks (Pontefract, 1991). Following good cleaning practices, the residues of detergents in milk should be minimal (≤ 2 ppm; Reybroeck, 1997), although owing to errors in the washing temperature, dosage, and inadequate post-rinse the concentration of these cleaning products can be higher, which may alter the organoleptic characteristics of milk (Merin *et al*., 1985; Dunsmore *et al*., 1985) and interfere in the activity of starter cultures in the industry (Guirguis & Hickey 1987; Petrova & Dimitrov 1993).

Moreover, only few studies in cow milk have evaluated the effect of detergents on the presence of positive results in microbial inhibitor tests, showing controversial results. Some authors (Zvirdauskiene & Salomskiene, 2007; Salomskiene *et al*., 2013) only found false-positive results at very high concentrations of alkaline detergents, equal or superior to the dose recommended by the manufacturers. However, Schiffmann *et al*. (1992) obtained non-compliant results at lower concentrations (0.01%), whereas Merin *et al*. (1985) for these concentrations did not obtain any positive results, although they employed a limited number of cleaning products and microbial methods. Furthermore, these studies focus on positive outcomes; there is no information about the effect of detergents on the detection capability of microbial methods.

Therefore, the goal of this study was to analyse the effect of detergents used in the cleaning of milking equipment on the performance of microbial tests for screening antibiotics in goat's milk.

Material and Methods

Microbial inhibitor tests

The microbial inhibitor tests were: Brilliant Black Reduction Test MRL (BRT MRL) (AiM Analytik in MilchProduktions-und Vertriebs-GmbH, Munich, Germany), Delvotest MCS (DSM Food Specialties, Delft, the Netherlands) and Eclipse 100 (Zeu-Inmunotec, Zaragoza, Spain). The tests were used according to each manufacturer's instructions. A negative control (antimicrobial-free milk) and a positive control (antimicrobial-free milk spiked with 4 µg/Kg of penicillin G) were included in each test. Visual interpretation of the test results was carried out independently by three trained technicians and was evaluated as "negative" (yellow) and "positive" (blue or purple).

Goat's Milk samples

Antimicrobial-free milk samples to be used as "negative milk" were obtained according to the requirements established by ISO13969/IDF183:2003. Therefore, mixed milk of 10 Murciano-Granadina goats in mid-lactation (more than 90 days and below 150 days postpartum) from the experimental flock of Universitat Politècnica de València (Valencia, Spain) was used. Animals had a good health status and did not receive any veterinary drugs before or during the experimental period. Moreover, goats were fed diets formulated and produced in the experimental feed processing plant of Universitat Politècnica de València using first-class raw materials without added antibiotics.

All milk samples were analysed to check the physico-chemical and hygienic quality parameters using MilkoScan 6000 (Foss, Hillerød, Denmark) to determine gross composition (fat, protein and total solids); somatic cell count (SCC) was obtained employing Fossomatic 5000 (Foss, Hillerød, Denmark); bacterial count (BC) was determined using Bactoscan FC (Foss, Hillerød, Denmark) and the pH value was measured by a conventional pHmeter (Crison, Barcelona, Spain).

Spiked milk samples: Detergents and Antibiotics

The detergents used for the study of their presence on the microbial test response were commercial detergents of the acid and alkaline type, which were added to the antibiotic-free goat's milk at concentrations of: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 ml/l for acid and 0, 0.5, 1, 2, 4, 6, 8, 10 ml/l for alkaline (Table 1). Each concentration was tested twelve times by microbial methods (BRT MRL, Delvotest MCS, and Eclipse 100).

To evaluate the effect of detergents on the detection capability of microbial screening methods for penicillins, the detection limits (DLs) for ampicillin, amoxicillin, cloxacillin and penicillin G were calculated according to ISO13969/IDF183:2003 specifications. To do so, two detergents were chosen, an acid one (Circoaction SF, Westfalia Surge Ibérica SL, Spain)

and a basic one (Circoaction AF, Westfalia Surge, Ibérica SL, Spain), which were then added to antibiotic-free goat's milk at the maximum detergent concentration, not showing interferences in the response of the microbial tests, nor significantly altering the pH values of milk (0.5 ml/l). Furthermore, goat's milk samples without detergents were used as control.

The goat's milk samples, with or without detergents, were spiked with eight different antibiotic concentrations (Table 2), prepared following the recommendations of the International Dairy Federation (IDF, 2003). The antibiotics selected for this study were supplied by Sigma-Aldrich (Madrid, Spain): amoxicillin (31586), ampicillin (A-9518), cloxacillin (C-9393), and penicillin G (PENNA). All the antibiotics standard solutions were prepared daily, and twelve repetitions of milk were analysed within four hours after spiking.

Statistical analysis

To evaluate the effects of the acid ($D_{AC,}$) or alkaline (D_{AK}) detergent on the response of the microbial inhibitor tests, the logistic regression model was used:

$$L_{ij} = \text{Logit}\,[P_{ij}] = \beta_0 + \beta_1\,[atb]_i + \beta_2\,D_{AC} + \beta_3\,D_{AK} + \varepsilon_{ij}$$

where: L_{ij}= Logit model; $[P_{ij}]$= probability for the response category (positive or negative); β_0= intercept; β_1, β_2, β_3= parameters estimated for the model; $[atb]_i$= effect of antibiotic concentration (n=8); D_{AC}= effect of the acid detergent; D_{AK}= effect of the alkaline detergent on the dummy variable (without detergent: D_{AC}= 0, D_{AK}= 0; acid detergent: D_{AC}= 1, D_{AK}= 0; alkaline detergent: D_{AC}= 0, D_{AK}= 1), ε_{ij} = residual error of model.

The detection limits (DLs) were calculated as an antibiotic concentration producing 95% positive results (ISO13969/IDF183:2003).

Statistical analysis was performed using Statgraphics Centurion XVI 5.1 (Statpoint Technologies, Inc, Warrenton, VA).

Results and Discussion

142 ***Effect of detergents in goat's milk on false-positive results of microbial screening tests***

143 The goat's milk samples presented an adequate physico-chemical quality (fat: 4.36 ± 0.12%,
144 protein: 3.61 ± 0.07%, dry matter: 14.24 ± 0.28%, and pH value: 6.66 ± 0.02) and hygienic-
145 sanitary parameters (SCC: 889 ± 79 x 10^3 cell/ml, and BC: 331 ± 51 x 10^3 cfu/ml) to be used
146 as antimicrobial-free milk.

147 The presence of the acid detergent in goat's milk did not affect the response of the microbial
148 inhibitor tests employed, the results were always negative. Moreover, the acid detergent
149 addition decreased the pH of milk samples, reaching values between 5.72 and 5.98 for the
150 highest concentration (2.5 ml/l) of the five detergents, being lower compared to the average
151 of pH cited for goat's milk (6.5-6.8; Park *et al*., 2007). These very low pH values can
152 simulate the effect of acid generation, which is produced as a consequence of the metabolism
153 of the microorganism, favouring the change in colour of the indicator present into the
154 medium test.

155 The alkaline detergent addition in goat's milk produced positive results at concentrations ≥2
156 ml/l (Table 3). Besides, at these concentrations, the pH of the milk samples was high,
157 reaching values of 9.82-10.80 for the highest concentrations tested, which could also inhibit
158 the growth of the microorganism and thus, prevent the colour change of the test indicator
159 system.

160 These results agree with Zvirdauskiene & Salomskiene (2007) and Salomskiene *et al*. (2013)
161 who studied the effects of various commercial detergents on the microbial test response in
162 cow milk, and found positive results at alkaline detergent concentrations equivalent to the
163 dose recommended by the manufactures and above. Although, it should be noted that these
164 high concentrations are very unlikely to be found in practice, even with poor cleaning
165 routines. Also, these authors did not find interferences due to the presence of acid detergents

166 in milk. At lower alkaline detergent concentrations (≤ 1,000 mg/l), Merin *et al*. (1985) and
167 Salomskiene *et al*. (2013) did not find any positive results for the Delvotest microbial test;
168 similar results than those obtained for most alkaline detergents tested in goat's milk (Table
169 3). However, Schiffmann *et al*. (1992) observed doubtful and positive results at very low
170 concentrations (0.01 mg/ml) using one acid detergent (Calgonit S) and a basic one (Calgonit
171 D) in different versions of the Brilliant Black Reduction Test (BRT).

172 In conclusion, only the presence of alkaline detergents in goat's milk at concentrations ≥
173 2ml/l, can produce positive results in microbial inhibitor tests. However, these amounts are
174 not reached if the rinsing of the milking equipment is carried out in an effective manner after
175 cleaning (Reybroeck, 1997).

176 ***Effect of detergents in goat's milk on the penicillin detection capability of microbial***
177 ***screening tests***

178 Table 4 shows the equations resulting from statistical analysis used to predict the positive
179 results for the penicillins and the detection limits (DL) of the microbial inhibitor tests. The
180 goodness-of-fit test shows that the experimental values are similar to those estimated by the
181 logistic model, suggesting a suitable adjustment of this model. The DLs calculated for
182 microbial tests in detergent-free milk were lower than those indicated by Sierra *et al*. (2009)
183 in goat's milk, which in most cases were closer to MRLs than those calculated in the present
184 study. These differences could be related to modifications carried out by manufacturers to
185 improve the sensitivity of these screening tests.

186 The presence of acid detergents in goat's milk did not affect or slightly increased the
187 sensitivity of the Delvotest MCS and Eclipse 100, showing lower DLs than those calculated
188 for detergent-free milk (Table 4). However, in the case of BRT MRL, the acid detergent
189 decreased the sensitivity to detect most penicillins in goat's milk (Table 4). For the alkaline

detergent, the DLs calculated for penicillins were below or equal to those obtained for detergent-free milk (Table 4), except for ampicillin in the Eclipse 100, which was slightly higher (4.3 *vs* 4 μg/l).

In spite of the statistical significant effect of the presence of detergents in goat's milk on the detection capability of the microbial tests, the DLs calculated were generally below MRLs established for each antimicrobial substance (Regulation EC 37/2010). Therefore, the presence of acid or basic detergent at concentrations equivalents to 0.05% does apparently not have any influence on the detection of raw milk containing penicillins above the safety levels when microbial screening tests are applied in the goat's milk quality control programmes.

However, is not known if a higher concentration of detergents in milk could have a serious effect on the detection capability of the methods employed to detect antibiotics in milk. No reference concerning the effect of detergents on the sensitivity of inhibitor tests for the penicillins or other antimicrobial agents was found; therefore the comparison of the results with other authors is not possible.

Conclusions

The response of microbial screening tests in goat's milk can be affected by the presence of alkaline detergents at high concentrations equal or greater than 2 ml/l. However, acid detergents did not produce any interference. Small amounts of acid and alkaline detergents in goats' milk (≤ 0.05 %) do not influence the sensitivity of the BRT MRL, Delvotest MCS and Eclipse 100 methods to detect penicillins. To avoid alterations in the milk quality and interferences in microbial screening tests employed in control programmes, the implementation of proper cleaning procedures to minimise the presence of detergent residues in milk is crucial.

Acknowledgements

This work forms part of the Project AGL 2009-11524 financed by the Ministry of Science and Innovation (Madrid. Spain) and the Generalitat Valenciana ACOMOP/2012/164 (Valencia, Spain). The authors are grateful to AiM Analytik in MilchProduktions-und Vertriebs-GmbH (Munich, Germany), DSM Food Specialties (Delft, Netherlands), ZEU-Inmunotec (Zaragoza. Spain) for their technological support.

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Table 1. Brand name, composition and recommended dose of acid and alkaline detergents.

Detergent	Brand Name	Composition (%)	Recommended dose (%)
Acid	Cid[†]	phosphoric acid/sulphuric acid (15-30/5-15%)	0.5-1
	105 Nifos[§]	phosphoric acid/sulphuric acid (25/5%)	0.5-1
	Grupacid[¶]	phosphoric acid/sulphuric acid (25/5%)	0.5-2
	Manocid[††]	ortophosphoric acid/nitric acid (25/10%)	0.5-1
	Circoaction SF[‡]	phosphoric acid/sulphuric acid (20-30/5-10%)	0.5-1
Alkali	Basix[†]	sodium hydroxide/sodium hypochlorite (5-15/5-15%)	0.5-1
	Circoaction AF[‡]	sodium hydroxide/sodium hypochlorite (<20/<10%)	0.5-1
	Clor FW[§]	sodium hydroxide/sodium hypochlorite (5/5%)	0.5-1
	Grupaclor[¶]	sodium hydroxide/sodium hypochlorite (5/10%)	0.5-1
	Manobactyl[††]	sodium hydroxide/sodium hypochlorite (5-10/5-10%)	0.5-1

[†]DeLaval International A.B., (Tumba, Sweden); [‡]GEA Farm Technologies Ibérica, S.L., (Barcelona, Spain); [§]OXA Chemical Specialties, CYGYC S.A., (Barcelona, Spain); [¶]Grupanor-Cercampo S.A., (Madrid, Spain); [††] Manovac S.L., (Valencia, Spain).

Table 2. Antibiotic concentrations used for the detection limit calculation of microbial inhibitor tests in goat's milk.

Antibiotic	Test	Concentrations (µg/Kg)							
Ampicillin	BRT MRL	0	1.0	1.3	1.6	1.9	2.2	2.5	2.8
	Delvotest MCS	0	1.3	1.6	1.9	2.2	2.5	2.8	3.1
	Eclipse 100	0	3.0	3.4	3.8	4.2	4.6	5.0	5.4
Amoxicillin	BRT MRL	0	0.7	1.0	1.3	1.6	1.9	2.2	2.5
	Delvotest MCS	0	0.7	1.0	1.3	1.6	1.9	2.2	2.5
	Eclipse 100	0	1.3	1.6	1.9	2.2	2.8	2.8	3.1
Cloxacillin	BRT MRL	0	5.0	7.0	9.0	11.0	13.0	15.0	17.0
	Delvotest MCS	0	5.0	7.0	9.0	11.0	13.0	15.0	17.0
	Eclipse 100	0	13.0	16.0	19.0	22.0	25.0	28.0	31.0
Penicillin G	BRT MRL	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4
	Delvotest MCS	0	0.4	0.6	0.8	1.0	1.2	1.4	1.6
	Eclipse 100	0	1.0	1.2	1.4	1.6	1.8	2.0	2.2

Table 3. Effect of the alkaline detergent concentrations in goat's milk on the positive results of microbial screening tests

Alkaline detergent	Test	Positive results (%)							
		Concentration ml/l							
		0	**0.5**	**1**	**2**	**4**	**6**	**8**	**10**
Basix	*pH*	*6.75*	*6.87*	*6.95*	*7.19*	*7.90*	*8.95*	*9.67*	*10.11*
	BRT MRL	0	0	0	0	100	100	100	100
	Delvotest MCS	0	0	0	0	100	100	100	100
	Eclipse 100	0	0	0	0	100	100	100	100
Circoaction AF	*pH*	*6.67*	*6.79*	*6.85*	*7.14*	*7.69*	*8.31*	*9.38*	*9.82*
	BRT MRL	0	0	0	0	100	100	100	100
	Delvotest MCS	0	0	8.3	16.7	25	100	100	100
	Eclipse 100	0	0	0	0	100	100	100	100
Clor FW	*pH*	*6.74*	*6.82*	*6.96*	*7.23*	*8.09*	*9.12*	*9.77*	*10.24*
	BRT MRL	0	0	8.3	100	100	100	100	100
	Delvotest MCS	0	0	0	0	100	100	100	100
	Eclipse 100	0	0	0	0	100	100	100	100
Grupaclor	*pH*	*6.76*	*6.87*	*7.03*	*7.41*	*8.94*	*9.86*	*10.41*	*10.80*
	BRT MRL	0	0	0	0	100	100	100	100
	Delvotest MCS	0	0	8.3	100	100	100	100	100
	Eclipse 100	0	0	16.7	100	100	100	100	100
Manobactyl	*pH*	*6.76*	*6.87*	*7.00*	*7.41*	*8.38*	*9.53*	*10.13*	*10.37*
	BRT MRL	0	0	0	0	100	100	100	100
	Delvotest MCS	0	0	0	0	100	100	100	100
	Eclipse 100	0	0	8.3	16.7	100	100	100	100

Table 4. Effect of the detergents in goat's milk on the penicillin detection capability of microbial inhibitor tests.

Antibiotic	Microbial Test	L=Logit[P]= $\beta_0+\beta_1[atb]+\beta_2D_{AC}+\beta_3D_{Ak}$				Goodness-of-fit test		Detection Limit (DL) µg/l		
		β_0	β_1	β_2	β_3	χ^2-value	p-value	DL_{DF}	DL_{AC}	DL_{AK}
Amoxicillin (MRL: 4µg/kg)	BRT MRL	-7.6680	4.4169	-	-	3.3615	0.3391	2.26	2.26	2.26
	Delvotest MCS	-11.7066	7.4322	-	1.8621	6.9116	0.0747	2.01	2.01	1.59
	Eclipse 100	-14.1769	6.0313	2.4226	2.1191	5.8541	0.1189	2.98	2.36	2.39
Ampicillin (MRL: 4µg/kg)	BRT MRL	-8.0068	4.6448	-1.1797	0.9588	2.5763	0.4616	2.25	2.71	2.13
	Delvotest MCS	-15.7129	6.2192	0.6260	3.7479	1.4916	0.6841	3.07	2.92	2.26
	Eclipse 100	-24.4051	6.8450	-	-2.0537	0.8155	0.8457	4	4	4.3
Cloxacillin (MRL: 30µg/kg)	BRT MRL	-9.8484	1.0550	-1.0595	0.5283	0.7530	0.8606	12.26	13.1	11.48
	Delvotest MCS	-12.4914	1.3079	-	-	0.7361	0.8646	12.11	12.11	12.11
	Eclipse 100	-19.4416	0.8936	2.2427	3.3505	1.5334	0.6746	23.67	22.12	23.4
Penicillin G (MRL: 4µg/kg)	BRT MRL	-5.7424	11.1144	-0.7414	1.2998	0.9408	0.8155	0.78	0.84	0.66
	Delvotest MCS	-11.6532	10.4337	4.0091	5.0684	0.3876	0.9427	1.52	0.93	0.8
	Eclipse 100	-8.1915	5.9682	-	-	3.3534	0.0589	1.9	1.9	1.9

L= ln (Probability (+)/1- Probability (+)); [atb]: antibiotic concentration; D_{AC}= effect of the acid detergent; D_{AK}= effect of the alkaline detergent on the dummy variable (detergent-free: D_{AC}= 0, D_{AK}= 0; acid detergent: D_{AC}= 1, D_{AK}= 0; alkaline detergent: D_{AC}= 0, D_{AK}= 1); DL_{DF}: detergent-free detection limit, DL_{AC}: acid detergent detection limit, DL_{AK}: alkaline detergent detection limit; MRL: Maximum residue limits; -: no significant differences $p > 0.05$.