Spanish Journal of Agricultural Research
13(1), e05-001, 9 pages (2015)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2015131-6522
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Analytical strategy for the detection of antibiotic residues in sheep and goat's milk

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Abstract

The use of antibiotics to treat mastitis and other infectious diseases in dairy sheep and goats is a widespread practice nowadays that can, when not properly applied, result in the contamination of the milk supply. Spanish legislation establishes the control of the presence of antibiotic residues in sheep and goat's milk using screening methods that detect, at least, beta-lactam drugs. Microbial inhibitor tests using *Geobacillus stearothermophilus* var. *calidolactis* and specific receptor-binding assays are most widely employed for this purpose. The detection rates of screening tests routinely used in Spain have been calculated considering the frequency of use of veterinary drugs commonly applied in ovine and caprine livestock to treat and prevent mastitis as well as the test sensitivity toward these substances at safety levels. In general, the use of a single test allows detecting 62.8-82.4% of the antibiotics employed. For sheep milk, the total detection range achieved with microbial tests was significantly higher than that reached with rapid receptor tests. However, no significant differences between the two types of methods were found when goat's milk was analysed. In both types of milk, the simultaneous use of two screening tests with a different analytical basis increases the total detection range significantly, reaching values ≥ 90% in some cases (81.5-90.1% for sheep and 84.7-92.6% for goats). However, the periodical use of screening tests able to detect quinolones, macrolides or aminoglycosides would be recommended to carry out more efficient screening and ensure the safety of milk and dairy products from sheep and goats.

Additional key words: antimicrobial agents; screening methods.

Abbreviations used: Fa (frequency of use of each antibiotic); MRL (maximum residue limit); PDO (protected designation of origin); PGI (protected geographical indication); SCC (somatic cell count); $SMRL_{t,a}$ (sensitivity of each test for each antibiotic at MRL equivalent concentration); TDRt (total detection rate for each screening test); TDRt1+t2 (total detection rate through the simultaneous use of two screening tests).

Citation: Beltrán, M. C.; Althaus, R. L.; Molina, A.; Berruga, M. I.; Molina, M. P. (2015). Analytical strategy for the detection of antibiotic residues in sheep and goat's milk. Spanish Journal of Agricultural Research, Volume 13, Issue 1, e05-001, 9 pages. http://dx.doi.org/10.5424/sjar/2015131-6522.
Received: 11 Jul 2014. **Accepted**: 26 Jan 2015.
http://dx.doi.org/10.5424/sjar/2015131-6522

Funding: This work forms part of the Project AGL2009-11524 financed by the Ministerio de Ciencia e Innovación (Madrid, Spain).
Competing interests: The authors have declared that no competing interests exist.

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Introduction

The Mediterranean basin is an important producer of sheep and goat's milk that is traditionally almost exclusively destined for the elaboration of dairy products, in particular cheese. Many of these products are elaborated according to stipulations concerning the protected designation of origin (PDO), the protected geographical indication (PGI), or traditional specialties guaranteed, internationally recognised, with high milk quality standards, especially in the case of products made from raw milk (Scintu & Piredda, 2007).

The quality of sheep and goat's milk has increased significantly in recent years, especially with regard to hygiene. In this sense, the establishment of Community legislation concerning the hygiene of foodstuffs of animal origin intended for human consumption (OJ, 2004) and the introduction of a payment system based on the quality of the milk from these species (Pirisi *et al.*, 2007) have decisively contributed to milk quality improvement.

The Spanish dairy sheep and goats sectors have become far more productive in the last decades thanks to greater specialisation and improved production facilities. Although the number of livestock farms has

been significantly reduced, the production of goat's milk has remained stable, while the production of sheep milk has increased considerably (MAGRAMA, 2013). In this context, the use of antimicrobials to treat and prevent infectious diseases in small dairy ruminants is a widespread practice that, if guidelines of good practices are not obeyed (IDF, 2013), can result in the contamination of the milk supply.

Antimicrobials should be applied under veterinary prescription using authorized products and respecting the dose, the routes of administration and withdrawal periods recommended by the manufacturers. However, the availability of drugs registered for the use in lactating dairy sheep and goats is quite limited, which conditions the off-label use of some antibiotics by veterinarians. Adequate withdrawal periods in milk from these species in off-label treatments are unknown in many cases which, therefore, increases the risk of residues of these substances in milk (Pengov & Kirbis, 2009).

The control of the presence of residues of veterinary agents in animal products for human consumption above maximum residue limits (MRLs) established by legislation (OJ, 2010) is mandatory in countries of the European Union (OJ, 2004). Antibiotic residues in milk and dairy products pose a risk to the health of consumers as they can cause allergic reactions in individuals sensitive to certain groups of antimicrobials, as well as generate antimicrobial resistance (Sanders *et al.*, 2011). Also, they are problematic for dairies as they can interfere in the fermentation processes required for the manufacture of certain milk products such as cheese and yoghurt (Berruga *et al.*, 2011).

For screening antibiotic residues in the milk supply, there are currently various analytical methods commercially available (ISO/IDF, 2010). Choosing a test depends on the control stage (farms, dairies, or laboratories) and also on the antibiotics applied to treat infectious diseases of dairy animals. Of all the screening methods available, microbial inhibitor tests using *Geobacillus stearothermophilus* var. *calidolactis* stand out due to their common use in control laboratories and rapid receptor tests for being widely used in farms and dairies given their swiftness of response.

Regarding sheep and goat's milk, Spanish regulation (BOE, 2011) establishes the control of the presence of antibiotic residues prior to the loading of milk into the tanker, if a risk for the consumer is suspected, using methods that detect at least beta-lactam drugs. In dairies, the control of the presence of beta-lactam residues must be carried out in all the tankers containing raw milk. Similarly, at control laboratories the use of screening tests able to detect at least beta-lactam drugs at MRL equivalent antibiotic concentration is also legally required. Whenever a non-compliant result is obtained, the milk sample should be re-tested applying another test with a similar detection profile and a different analytical basis.

Mastitis is undoubtedly the infectious disease most frequently treated with antibiotics in dairy livestock. Thus, mastitis treatments are applied in the lactation- and dry-off periods, being the main cause of the presence of antibiotic residues in cow milk (Fabre *et al.*, 1995). In ovine and caprine species, there are no studies on the causes of antibiotic residues in milk. However, Gonzalo *et al.* (2010) detected a significant effect of some mastitis-related aspects such as elevated somatic cell counts (SCCs) in milk, and the use of the dry-off therapy on the occurrence of antibiotic residues in sheep milk, especially when antibiotics are administered in a discontinued manner.

In dairy sheep and goats, mastitis is treated by veterinarians during lactation as well as in dry-off therapy, using primarily beta-lactam drugs, while macrolides constitute the second most important group of antimicrobials applied (Berruga *et al.*, 2008). Therefore, substances belonging to these two antibiotic families are the most probable residues in raw milk from these species. Other substances such as tetracyclines and quinolones are less commonly used in mastitis treatments; however, they are usually employed in other respiratory, digestive and reproductive diseases requiring antibiotic therapy in small ruminants. Therefore, in order to carry out effective screening of raw milk from sheep and goats, it would be desirable to have analytical methods available to detect the most frequent drugs currently used in veterinary medicine.

For this reason, the objective of this study was to evaluate an analytical strategy based on the use of different commercially screening methods routinely employed in Spain to detect antibiotic substances most commonly applied in a simple and economic manner.

Material and methods

Milk samples

Antibiotic-free milk samples were obtained from the experimental flocks of Manchega sheep of Universidad de Castilla-La Mancha (Albacete, Spain), and Murciano-Granadina goats of Universitat Politècnica de València (Valencia, Spain). Animals had a good health status and had not received any veterinary drugs, neither before nor along the experimental period. Neither was medicated feed used in their diet.

Individual milk samples (200 mL) were collected in the mid-lactation period from 40 sheep (more than 60

days and below 90 days postpartum) and 40 goats (more than 90 days and below 150 days postpartum). All milk samples were analysed for gross composition (MilkoScan 6000, Foss. Hillerød, Denmark), somatic cell count (Fossomatic 5000, Foss), total bacterial count (Bactoscan FC, Foss), and pH value to check their suitability to be used as "negative milk" according to the IDF recommendations (ISO/IDF, 2003a,b).

Antibiotic screening tests

Microbial inhibitor tests and receptor-binding assays most commonly used in Spain for screening antibiotics in sheep and goat's milk were employed in this study.

The microbial inhibitor tests used were the BRT MRL (Analytik in Milch Produktionsund Vertriebs, GmbH, Munich, Germany), Delvotest MCS SP-NT (DSM Food Specialties. Delft, the Netherlands), Delvotest MCS Accelerator (DSM Food Specialties), and Eclipse 100 (Zeulab, Zaragoza, Spain).

The receptor-binding assays were the Betastar Combo test (Neogen Corporation, Lansing, MI, USA), the Charm MRL BLTET test (Charm Sciences, Inc., Lawrence, MA, USA), the SNAP Betalactam test (IDEXX Laboratoires, Westbrook, ME, USA), the SNAP Tetracycline test (IDEXX Laboratories), and the Twinsensor^{BT} test (Unisensor, Liege, Belgium), which employ binding reagents and have similar reaction mechanisms. The Betastar Combo, Charm MRL BLTET and Twinsensor^{BT} tests allow to simultaneously detect both beta-lactam and tetracycline antibiotics in milk samples, and the SNAP tests used, namely SNAP Betalactam, and SNAP Tetracycline, are specific for beta-lactams and tetracyclines, respectively.

All tests were conducted according to the manufacturer's instructions. The microbial test results were classified as positive or negative visually by three trained technicians who carried out the classification independently from each other, *i.e.* at least two observations had to coincide to obtain the final result, while the receptor-binding test results were classified using specific devices provided by the manufacturers. However, in the case of the Delvotest MCS Accelerator, the reading was performed only instrumentally, using the Delvotest Accelerator device.

Antimicrobials and spiked milk samples

Antimicrobials most commonly employed by veterinarians to treat and prevent mastitis in dairy sheep and goats were selected for this study. In agreement with Berruga *et al.* (2008) who surveyed veterinarians for information on antimicrobial treatments most commonly applied in Spain for the antibiotic therapy in small dairy ruminants, a total of 26 substances was investigated: amoxicillin (A8523), ampicillin (A9518), benzylpenicillin (PENNA), cloxacillin (C9393), cefalonium (32904), cefapirin (43989), cefazolin (C5020), cefoperazone (32426), cefquinome (32472), ceftiofur (34001), cephalexin (C4895), enrofloxacin (33699), erythromycin (E6376), gentamicin (G3632), lincomycin (31727), marbofloxacin (34039), neomycin (N1876), oxytetracycline (O4636), spiramycin (59132), streptomycin (S6501), sulfadiazine (S6387), sulfametazine (S5637), tetracycline (T3258) and tylosin (T6271) were supplied by Sigma-Aldrich Química, S.A. (Madrid, Spain). Cefacetrile, not commercially available, were kindly provided by Fatro S.p.A. (Bologna, Italy).

Commercial drugs were stored and handled as indicated by the manufacturers. For use, antimicrobials were dissolved (1 mg/mL) at the time when analyses were carried out to avoid problems related to instability.

Spiked milk samples were prepared following the recommendations of the International Dairy Federation (ISO/IDF, 2003a,b), and tested simultaneously by the different screening tests immediately after spiking. For each drug, 60 replicates of antibiotic-free milk spiked at MRL-equivalent antibiotic concentration were made using sheep and goat's milk, respectively. All antimicrobial substances were tested by the four microbial inhibitor tests considered. For rapid receptor tests only beta-lactams and tetracyclines were analysed because they were designed specifically for the detection of these drugs.

The test sensitivity was calculated for each antibiotic substance as the percentage of positive results on the total of milk samples analyzed.

Calculation of the total detection rate for screening tests

Taking into account the frequency of use of each "a" antimicrobial substance (Fa), calculated from data provided by Berruga *et al.* (2008), and the "t" test sensitivity for each antibiotic at MRL equivalent concentration (SMRLt,a), the detection rates of each screening test were calculated.

$$DR_{t,a}(\%) = F_{a\,x}\,SMRL_{t,a} \qquad [1]$$

Subsequently, the total detection rate for each screening test (TDRt) was calculated according to the following mathematical expression:

$$TDR_t = \sum_{a=1}^{a=n} DR_{t,a} \qquad [2]$$

Calculation of the total detection rate through the simultaneous use of two screening tests

The total detection rate resulting from the simultaneous use of two screening tests (TDR_{t1+t2}) was calculated by adding the detection rate of the screening method presenting the highest sensitivity for each antibiotic substance as shown in the following expression:

$$TDR_{t1+t2} = \sum_{a=1}^{a=n} DR_{t1,a}(t1/t2) + \sum_{a=1}^{a=n} DR_{t2,a}(t2/t1) \quad [3]$$

where $DR_{t1,a}(t_1/t_2) = F_a\ SMRL_{t1,a}$: detection rate presented by test t_1 for a given antibiotic a, not detected by test t_2 (or the sensitivity of t_2 is below that of t_1); $DR_{t,a}(t_2/t_1) = F_a\ SMRL_{t2,a}$: detection rate presented by test t_2 for a given antibiotic a, not detected by test t_1 (or the sensitivity of t_1 is below that of t_2).

Statistical analysis

The total detection rates obtained for microbial inhibitor tests and receptor-binding assays were compared through the non-parametric Mann-Wilcoxon test in order to establish significant differences ($p<0.05$) between them or their possible combinations. Statistical analysis was performed using the Statgraphics software (Statgraphics Centurion XVI).

Results and discussion

Table 1 summarizes the frequency of use of antibiotics most commonly applied in the mastitis treatments of dairy sheep and goats, and the microbial test sensitivity for each antibiotic substance. Information related to rapid receptor tests is presented in Table 2.

As shown in Table 1, although microbial screening tests have the same analytical basis they display different sensitivities for the detection of antibiotics. Thus, for example, molecules such as benzylpenicillin, cefalonium or tylosin are detected by all microbial inhibitor tests (sensitivity ≥ 95%), while there are molecules that are only detected by some methods and not by others. Thus, for example, the BRT MRL test has lower specificity for ceftiofur and cephalexin than the Delvotest MCS SP-NT, Delvotest MCS DA and Eclipse 100 tests; however, it is the only microbial test able to detect erythromycin and gentamicin at MRL equivalent concentration in sheep and goat's milk.

Similar results were reached with the receptor-binding assays studied. Of all the beta-lactams and tetracyclines used in dairy sheep and goats, some molecules, for instance, ampicillin, benzylpenicillin and cefoperazone, among others, are detected by all the rapid receptor tests considered (Table 2). On the contrary, there are substances that are only detected by some tests and not by others, as, for example cloxacillin, which is detected by the Betastar Combo, SNAP and Twinsensor^{BT} tests but only to a very low extent by the Charm MRL BLTET test.

Using Eq. [1] presented in the Materials and Methods section, the total detection rate for each screening test (TDR_t) was calculated and summarized in Table 3. It should be noted that SNAP Betalactam and SNAP Tetracycline tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously.

As shown in Table 3, of the four microbial inhibitor tests to detect antibiotics in milk, the BRT MRL test has the highest total detection rate (sheep: 82.1%; goats: 82.3%) compared with the Delvotest MCS SP-NT, Delvotest MCS DA and Eclipse 100 tests. In this respect it should be mentioned that the BRT MRL test uses Mueller Hinton as culture medium and black brilliant as redox indicator unlike the other three tests containing Plate count agar and bromocresol purple as acid-base indicator. These differences could be related to the greater sensitivity towards some antimicrobial substances belonging to families other than beta-lactams.

Concerning the rapid receptor tests evaluated, the SNAP test presented a higher total detection rate than the Betastar Combo, Charm MRL BLTET, and Twinsensor^{BT} tests (Table 3) due to its greater sensitivity to cephalexin, while the Charm MRL BLTET test displayed the lowest total detection rate given its low sensitivity towards cloxacillin. It should be noted that the receptor-binding assays used in this study are designed for the specific detection of beta-lactams and tetracyclines and therefore, drugs belonging to other groups of antibiotics cannot be detected by these tests; which explains the relatively low detection percentages obtained (sheep: 62.8-74.9%; goats: 71.6-82.4%) with this test type when all antimicrobials are considered. Therefore, from a food safety point of view, the application of one test only seems insufficient as an appreciable percentage of antibiotic residues remains undetected and could thus reach the consumer.

When comparing the total detection rates achieved in sheep milk with microbial and rapid receptor screening tests, respectively, through the Mann-Wilcoxon contrast test, significant differences between the two

Table 1. Sensitivity of microbial screening tests at Maximum Residue Limits (MRL) equivalent antibiotic concentration

Antimicrobials	Sheep milk					Goat milk				
	F[1]	SMRL[2] (%)				F	SMRL (%)			
		BRT MRL	Delvotest SP-NT	Delvotest DA	Eclipse 100		BRT MRL	Delvotest SP-NT	Delvotest DA	Eclipse 100
Beta-lactams										
Benzylpenicillin	29.10	100	100	100	100	30.00	100	100	100	100
Amoxicillin	15.00	100	100	100	100	20.00	100	100	97	95
Cloxacillin	14.20	100	100	100	100	10.00	100	100	100	97
Ceftiofur	3.10	45	100	100	100	3.60	47	80	73	92
Ampicillin	2.40	100	100	100	95	2.10	100	100	100	100
Cephalexin	2.40	47	100	100	100	1.40	35	100	100	100
Cefquinome	1.60	0	0	0	0	0.70	33	28	12	3
Cefoperazone	1.60	58	77	32	15	0.70	48	83	58	28
Cefazolin	0.80	100	100	100	100	0.70	100	100	100	100
Cefalonium	0.80	100	100	100	100	0.70	100	100	100	100
Cefapirin	0.80	100	100	100	100	0.70	100	100	100	100
Cefacetrile	0.80	100	100	100	100	0.70	100	100	100	100
Macrolides										
Erytromycin	6.70	96	17	8	5	5.10	100	45	10	17
Tylosin	6.40	100	100	100	100	3.20	100	100	100	95
Spiramicin	3.90	0	0	0	0	2.20	0	0	0	0
Lincomycin	1.80	82	23	68	5	1.20	55	40	80	3
Tetracyclines										
Oxitetracycline	2.30	0	5	25	32	8.20	38	20	30	47
Tetracycline	0.50	0	3	0	0	1.60	0	5	5	5
Quinolones										
Enrofloxacin	3.80	2	0	0	0	3.60	0	0	0	0
Marbofloxacin	1.20	0	0	0	8	0.70	0	0	0	0
Aminoglycosides										
Streptomicin	0.30	16	0	0	8	0.40	25	5	5	25
Gentamicin	0.30	100	17	15	5	1.20	97	13	5	5
Neomicin	0.10	100	100	100	70	1.20	100	100	100	65
Sulphonamides										
Sulfadiazine	0.10	60	100	100	100	0.00	78	100	100	100
Sulfametazine	0.10	80	33	55	93	0.00	70	48	65	90

[1]F: Frequency of use of antimicrobials in mastitis treatments calculated from data provided by Berruga *et al.* (2008); [2]SMRL: Sensitivity of screening tests at Maximum Residue Limits (MRL) equivalent antibiotic concentration.

types of assays were found (W=16.0, p=0.030), *i.e.* a broader spectrum of detection was achieved with microbial screening tests (77.2-82.1 *vs* 62.8-74.9%, respectively). This is due to the fact that mastitis therapy in sheep makes an appreciable use of macrolides using substances such as erythromycin (6.7%), tylosin (6.4%) and lincomycin (1.8%), some of which are detected by the microbial screening tests assessed but not by the rapid receptor tests. However, the application of the Mann-Wilcoxon test to compare the total detection ranges obtained in both groups of the screening tests in goat's milk did not show significant differences (W=0.3806, p=0.7166), indicating that they could be used interchangeably with similar levels of detection. For this reason, when a rapid response is required (*i.e.* the control of antibiotic residues in farms and dairies), the use of a receptor-binding assay would be appropriate, while in case of a large number of milk samples to be checked, the use of the microbial test would be recommendable and also more economical.

Concerning the simultaneous use of two screening methods trying to improve the percentage of total detection of antibiotics in sheep and goat's milk, the results obtained for the different combinations possible are presented in Tables 4 and 5.

As shown in Table 4, the simultaneous use of two microbiological screening methods in sheep milk made the detection of a range between 78.9 and 85.9% of the molecules considered possible, presenting no statistically significant differences with respect to that ob-

Table 2. Sensitivity of receptor-binding assays at Maximum Residue Limits (MRL) equivalent antibiotic concentration

Antimicrobials	Sheep milk					Goat milk				
	F[1]	SMRL[2] (%)				F	SMRL[2] (%)			
		Betastar Combo	Charm MRL	SNAP	Twin-sensor		Betastar Combo	Charm MRL	SNAP	Twin-sensor
Beta-lactams										
Benzylpenicillin	29.10	100	100	100	100	30.00	100	100	100	100
Amoxicillin	15.00	98	95	98	97	20.00	100	95	100	100
Cloxacillin	14.20	100	18	100	100	10.00	100	15	100	100
Ceftiofur	3.10	92	100	100	100	3.60	90	100	100	100
Ampicilin	2.40	100	97	100	100	2.10	100	97	100	100
Cefalexin	2.40	0	100	100	0	1.40	5	100	100	0
Cefquinome	1.60	100	100	100	100	0.70	100	100	100	100
Cefoperazone	1.60	100	100	100	100	0.70	100	100	100	100
Cefazolin	0.80	95	100	100	100	0.70	97	100	100	100
Cefalonium	0.80	100	100	95	100	0.70	100	100	100	100
Cephapirin	0.80	100	100	100	100	0.70	100	100	100	100
Cefacetrile	0.80	100	100	100	100	0.70	100	100	100	100
Tetracyclines										
Oxitetracycline	2.30	97	100	100	100	8.20	100	100	100	100
Tetracycline	0.50	97	100	100	97	1.60	100	95	100	100

[1]F: Frequency of use of antimicrobials in mastitis treatments calculated from data provided by Berruga *et al.* (2008); [2]SMRL: Sensitivity of screening tests at MRL equivalent antibiotic concentration.

tained with the use of a single screening test belonging to this group (W=20.5, p=0.087). Similarly, the combination of two rapid screening tests based on the use of specific receptors neither increased the detection range of antibiotics in sheep milk (W=20.0, p=0.1056) with respect to the use of a single test. Therefore, it can be concluded that the simultaneous use of two methods having the same analytical basis does not improve the detection of antibiotic substances commonly used in dairy ovines.

On the other hand, the combination of two methods with a different analytical basis, *i.e.* a microbial test together with a receptor-binding test, resulted in a broader detection spectrum (81.5-90.1%) which was statistically significant with respect to that obtained with a single microbial or a rapid test (W=15.6812, p=0.0005) allowing, therefore, a more efficient control of antibiotic residues in sheep milk.

In goat's milk, the simultaneous use of two microbial screening tests (Table 5) does not lead to an improvement of the total detection ranges in comparison to the use of a single test (W=21.0, p=0.069) leaving a percentage of undetected residues ranging from 14.4 to 20.1%. Similarly, when applying the Mann-Wilcoxon test to compare the total detection rates obtained by the application of a single rapid receptor test (Table 3) with those calculated when two receptor-binding assays were used simultaneously (Table 5), significant differences were not found (W=21.0, p=0.069). Therefore, a combination of two rapid receptor tests does not increase the detection range, and a percentage of undetected substances between 17.6 and 20.1% remains.

On the contrary, when the detection ranges achieved through the simultaneous use of receptor-binding assays and microbial inhibitor tests are calculated (84.7 to 92.6%), it can be observed that the total detection rates are higher than those calculated when using only a rapid receptor test (ranging between 71.6 and 82.4%, W=93.0, p=0.001) or only a microbial method (77.4 and 82.3%, W=96.0, p=0.0004). Hence, the application of two screening tests with a different analytical basis leads to a significant improvement in milk safety as a greater percentage of the potential antibiotic residues in milk is detected.

Table 3. Total detection rate (TDR) of antibiotics reached by screening tests in sheep and goat's milk

	TDR (%)	
	Sheep	Goat
Microbial tests		
BRT MRL	82.1	82.3
Delvotest MCS SP-NT	78.9	79.1
Delvotest MCS DA	78.8	77.4
Eclipse 100	77.2	77.4
Rapid receptor tests		
Betastar Combo	72.2	79.5
Charm MRL BLTET	62.8	71.6
SNAP	74.9	82.4
Twinsensor[BT]	72.4	79.8

Table 4. Detection rates of antibiotics reached with the simultaneous use of two screening tests in sheep milk

	Charm	SNAP	Twinsensor	BRT	Delvotest	Delvotest DA	Eclipse
Betastar	74.5	74.9	72.6	88.5	83.4	83.5	82.2
Charm		84.9	74.8	90.1	83.5	83.6	85.4
SNAP			74.9	90.1	83.5	83.6	82.3
Twinsensor				88.8	83.4	83.6	81.5
BRT					85.5	85.7	85.9
Delvotest						80.1	79.5
Delvotest DA							78.9

Table 5. Detection rates of antibiotics reached with the simultaneous use of two screening tests in goat's milk

	Charm	SNAP	Twinsensor	BRT	Delvotest	Delvotest DA	Eclipse
Betastar	81.2	82.4	79.9	90.3	88.2	86.8	84.7
Charm		82.4	81.2	90.7	88.5	86.5	84.7
SNAP			82.4	92.6	88.6	87.2	88.1
Twinsensor				91.7	87.0	87.0	86.1
BRT					84.7	84.5	85.6
Delvotest						80.4	81.6
Delvotest DA							79.9

Discussion

In general, microbial screening tests using *G. stearothermophilus* present a high sensitivity for the detection of beta-lactam antibiotics in sheep as well as goat's milk. Thus, the detection rates for this group of antimicrobials are higher than those obtained for families other than beta-lactams, especially for drugs belonging to the tetracycline and quinolone groups that were not detected at safety levels by any of the microbial tests considered. These results were in agreement with those obtained by other authors in milk from these species several years ago (Althaus *et al.*, 2003; Molina *et al.*, 2003; Montero *et al.*, 2005; Sierra *et al.*, 2009a,b), although it is noteworthy that some drugs such as neomycin, tylosin and sulfadimethoxine can be more easily detected by the new versions of microbial test commercially available (Beltrán *et al.*, 2015).

The rapid receptor tests considered in this study were designed for the specific detection of beta-lactam and tetracycline residues in milk presenting detection capabilities equal to or below the MRLs for most beta-lactams and tetracyclines employed by veterinarians (Beltrán *et al.*, 2013, 2014a,b) and, consequently, higher detection rates for these antibiotics in milk are obtained.

It should be kept in mind that Spanish legislation (BOE, 2011) currently centers the analytical strategy for the control of the presence of antibiotics in sheep and goat's milk mainly on the detection of beta-lactams. Therefore, the effectiveness of the analytical strategy currently applied in the sector for screening antibiotics in raw milk from sheep and goats, allows achieving elevated detection ranges, above 90% in most cases, both in dairy sheep (microbial tests: 92.8-97.4%, rapid tests: 82.9-99.6%) and goats (microbial tests: 94.9-98.1%, rapid tests: 86.6-100%), respectively, owing to the higher sensitivity of these screening tests for beta-lactam drugs.

In the case of cow milk, Spanish legislation (BOE, 2007) centers the control of the presence of beta-lactam and tetracycline residues on obligatory checks of all tankers used by the dairy industry for the presence of beta-lactams. In the case of tetracyclines, these checks are obligatory in one out of five tankers, assuring, however, that all routes are checked on a monthly basis.

If the specific detection of tetracycline residues were included as a requirement for screening antibiotics in sheep and goat's milk, the effectiveness of the analytical strategy would decline slightly for sheep (microbial tests: 89.6-94.5%, rapid tests: 83.4-99.6%) and goat's milk (microbial tests: 87.3-89.3%, rapid tests: 88.2-100%), respectively, because, although the receptor-binding assays are able to detect oxitetracycline and tetracycline at their respective MRLs, microbial screening tests are less sensitive to these substances at safety levels and, therefore, the total detection rate is reduced.

When considering all the substances potentially present in milk as residues, the decline in the effectiveness of the current analytical strategy is more pronounced, obtaining a percentage of undetected residues ranging from 9.9 to 18.5% for sheep, and from 7.4 to 15.3% for goats, respectively, mainly due to drugs belonging to the quinolone and macrolide families as the screening tests present a lower sensitivity towards these substances. Thus, the periodical implementation

of screening tests more sensitive to these substances would be convenient to increase the detection spectrum and, logically, minimize the risks stemming from the presence of these residues in milk. Thus, for example the application of the *Bacillus subtilis* bioassay would complement antibiotic coverage achieved by microbial inhibitor tests using *G. stearothermophilus*, detecting substances such as erythromycin, enrofloxacin and spiramycin at concentrations much closer to their respective MRLs (Nagel *et al.*, 2012). Also, the periodic use of specific rapid methods for the detection of aminoglycosides and quinolones could be an alternative to increase the detection spectrum.

In conclusion, the simultaneous use of two screening tests with a different analytical basis allows achieving a broader coverage of the antimicrobials used to treat and prevent mastitis in dairy sheep and goats which pose the greatest risk of appearing in milk. However, taking in to account that antibiotic agents such as quinolones, macrolides or aminoglycosides are not detected by the screening tests assessed and are also used to treat mastitis or another respiratory, reproductive or digestive diseases, the improvement of the analytical strategy through the periodical implementation of screening tests able to detect these substances at safety levels, would be recommended. Besides establishing a suitable control strategy, it should not be forgotten that the application of a code of good dairy farming practices concerning the use of veterinary drugs should be adhered to in order to avoid the presence of residues in milk and dairy products

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