UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Ph. D. Thesis

Analytical strategy for the detection of antibiotic residues in milk from small ruminants

Mª Carmen Beltrán Martínez

Supervisors:

Dr. Mª Pilar Molina Pons
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Departamento de Ciencia Animal
Instituto de Ciencia y Tecnología Animal
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**Mª PILAR MOLINA PONS, CATEDRÁTICA DE UNIVERSIDAD DEL DEPARTAMENTO DE CIENCIA ANIMAL DE LA UNIVERSITAT POLITÈCNICA DE VALÈNCIA
Y
RAFAEL LISANDRO ALTHAUS, PROFESOR DEL DEPARTAMENTO DE CIENCIAS BÁSICAS DE LA FACULTAD DE CIENCIAS VETERINARIAS DE LA UNIVERSIDAD NACIONAL DEL LITORAL (REPUBLICA ARGENTINA)**

INFORMAN:

Que la Tesis Doctoral titulada "Estrategia analítica para la detección de residuos de antibióticos en leche de pequeños rumiantes" ha sido realizada por Dña. Mª Carmen Beltrán Martínez en el Departamento de Ciencia Animal bajo su dirección y que, una vez revisado y comprobado el trabajo, consideran que reúne los requisitos necesarios para la obtención del grado de Doctor por lo que autorizan su presentación.

Y para que así conste firman el presente informe en Valencia, a diez de septiembre de 2014

Dra. Mª Pilar Molina Pons   Dr. Rafael Lisandro Althaus

A mi querido esposo, Jaime y

a nuestros maravillosos hijos, Marta y Jaume

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Summary

In Mediterranean countries, sheep and goat's milk production has traditionally been destined for the manufacture of cheese, often as raw milk. Cheese quality is closely related to milk composition but also to hygienic aspects such as somatic cell count, bacteriology or presence of antibiotic residues, currently regulated by European legislation.

The implications of the presence of antibiotic residues in milk as a result of veterinary treatments include negative effects on consumer's health such as allergies or antibiotic resistance and problems on the manufacturing processes of fermented products. For the screening of milk samples for antimicrobial residues, there are various methods available, microbial inhibitor tests and assays based on specific receptors, both widely used, especially in farms, the dairy industry and control laboratories. Screening methods have been validated for the use in raw milk from cows, but information on the performance of these tests in sheep and goat's milk is rather limited.

The aim of this study was to evaluate the performance of some microbial and receptor-binding screening tests to detect antibiotics in sheep and goat's milk according to Commission Decision 2002/657/EC to determine their suitability to monitor the presence of antibiotic residues in milk and establish the most convenient analytical strategy in Spain.

The Detection capability (CCβ) of microbial screening tests, the BRT MRL, the Delvotest MCS SP-NT, the Delvotest MCS Accelerator and the Eclipse 100, was at or below the maximum residue limits (MRLs) for most beta-lactam antibiotics assessed and other non-beta-lactam drugs such as neomycin, tylosin, sulfadiazine and sulfadimethoxine. However, they were less sensitive in the detection of quinolones and tetracyclines at safety levels. When individual milk samples were analysed, microbiological tests showed a higher occurrence of non-compliant results in sheep milk than in goat's milk, being related in all cases to an elevated somatic cell count (SCC).

The microbiological system consisting of two complemetary microtiter plates containing *Geobacillus stereathermophilus* var. *calidolactis* and *Bacillus subtilis*, respectively, allows improving the detection level in sheep milk with respect to the use of a single commercial test using *G. stearothermophilus*, detecting some quinolone and macrolide substances more closely related to their respective MRLs.

The rapid receptor-binding assays (the Betastar Combo, the Charm MRL BLTET, the SNAP Betalactam, the SNAP Tetracycline and the Twinsensor[BT]) were able to detect most beta-lactams and tetracyclines at or below MRLs (CCβ ≤ MRL). A higher specificity of the rapid receptor tests was obtained in all cases even when individual milk samples were analysed. Only the Twinsensor[BT] test presented non-

compliant results when antibiotic-free milk samples from individual animals were analysed, especially in the last weeks of lactation. No cross-reactions were found when drugs belonging to antimicrobial groups other than beta-lactams or tetracyclines were present in milk. Azidiol, used as a preservative, had no effect on the performance of the rapid receptor tests. Moreover, differences between the visual and instrumental classification of the test results were not found.

Taking into account the frequency of use of antibiotics commonly employed in Spain and the screening test sensitivity at MRLs equivalent to antibiotic concentrations, total detection rates have been calculated. 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 tests 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.

However, antibiotics such as enrofloxacin, marbofloxacin, spiramycin, and streptomycin also used to treat mastitis and other infectious diseases could not be detected by the screening tests assessed. Therefore, the improvement of the analytical strategy through the periodical implementation of screening tests able to detect these substances at safety levels would be recommended.

Resumen

En los países mediterráneos, la producción de leche de oveja y de cabra se ha destinado tradicionalmente a la fabricación de queso, a menudo a partir de leche cruda. La calidad del queso está estrechamente relacionada con la composición de la leche pero también con aspectos higiénicos actualmente regulados por la legislación europea, como el recuento de células somáticas, la bacteriología o la presencia de residuos de antibióticos.

Las implicaciones de la presencia de residuos de antibióticos en la leche, como resultado de los tratamientos veterinarios, incluyen efectos negativos sobre la salud del consumidor tales como alergias o resistencias bacterianas a los antibióticos y, también, problemas en la fabricación de productos lácteos fermentados. Para la detección de residuos de antibióticos en las muestras de leche existen varios métodos de cribado disponibles siendo los métodos basados en la inhibición del crecimiento microbiano y los ensayos a base de receptores específicos los más utilizados en granjas, industrias lácteas y laboratorios de control. Estos métodos de cribado han sido validados para su uso en leche cruda de vaca, pero la información sobre los resultados de estas pruebas en leche de oveja y cabra es bastante limitada.

El objetivo de este estudio ha sido evaluar el funcionamiento de algunos métodos microbiológicos y ensayos rápidos de receptores para la detección de antibióticos en leche de oveja y cabra según la Decisión 657/2002/CE, con objeto de determinar su idoneidad para controlar la presencia de residuos de antibióticos en la leche y establecer la estrategia analítica más conveniente en España.

La Capacidad de detección (CCβ) de los métodos microbiológicos de cribado, BRT MRL, Delvotest MCS SP-NT, Delvotest MCS Accelerator y Eclipse 100, fue igual o inferior a los límites máximos de residuos (LMRs) establecidos por la legislación europea para la mayor parte de antibióticos betalactámicos estudiados y otros no betalactámicos como neomicina, tilosina, sulfadiazina y sulfadimetoxina. Sin embargo, fueron menos sensibles para detectar quinolonas y tetraciclinas a sus respectivos niveles de seguridad. Cuando se analizaron muestras de leche individuales, los tests microbiológicos presentaron una mayor ocurrencia de resultados no conformes para la leche de oveja que estuvo relacionada en todos los casos, con un recuento elevado de células somáticas (RCS).

El Sistema microbiológico formado por dos microplacas complementarias basadas en la utilización de *Geobacillus stearothermophilus* var. *calidolactis* and *Bacillus subtilis*, respectivamente, permite mejorar los niveles de detección en leche de oveja con respecto al uso de un único test comercial empleando *G. stearothermophilus*, al detectar algunas sustancias del grupo de las quinolonas y de los macrólidos a concentraciones más próximas a sus respectivos LMRs.

Los ensayos rápidos de receptores (Charm MRL BLTET, Betastar Combo, SNAP Betalactam, SNAP Tetracycline y Twinsensor[BT]) fueron adecuados para detectar la mayor parte de betalactámicos y tetraciclinas a concentraciones iguales o inferiores a los LMRs (CCβ ≤ MRL). La especificidad de los tests de receptores fue elevada en todos los casos incluso cuando se analizaron muestras de leche individuales. Únicamente el test Twinsensor[BT] presentó resultados no conformes cuando se analizaron muestras libres de antibióticos procedentes de animales individuales, especialmente en las últimas semanas de lactación. No se observaron reacciones cruzadas con la presencia de sustancias pertenecientes a otras familias de antibióticos distintas a las de betalactámicos y tetraciclinas en la leche. El conservante azidiol no tuvo ningún efecto sobre la respuesta de los tests de receptores. Además, no se encontraron diferencias entre la interpretación visual y la instrumental de los resultados.

Teniendo en cuenta la frecuencia de uso de los antibióticos comúnmente empleados en España y la sensibilidad de los métodos a una concentración de antibiótico equivalente al LMR, se calcularon los ratios totales de detección en el cribado. En general, el uso de un solo test permite detectar 62.8-82.4 % de los antibióticos empleados. Para la leche de oveja, el rango total de detección alcanzado con los métodos microbiológicos fue significativamente mayor que el alcanzado con las pruebas rápidas de receptores. Sin embargo, no se encontraron diferencias significativas entre los dos tipos de tests cuando se analizó leche de cabra. En ambos tipos de leche, el uso simultáneo de dos tests de cribado con diferente base analítica, incrementó significativamente el rango total de detección alcanzando valores ≥ 90 % en algunos casos.

Sin embargo, antibióticos tales como enrofloxacina, marbofloxacina, espiramicina y estreptomicina que también se utilizan para tratar la mamitis y otras enfermedades infecciosas del ganado, no son detectadas por los tests de cribado evaluados. Por tanto, la mejora de la estrategia analítica a través de la aplicación periódica de pruebas capaces de detectar la presencia de estas sustancias a los niveles de seguridad establecidos sería recomendable.

Resum

En els països mediterranis, la producció de llet d'ovella i de cabra s'ha destinat tradicionalment a la fabricació de formatge, sovint a partir de llet crua. La qualitat del formatge està estretament relacionada amb la composició de la llet però també amb aspectes higiènics actualment regulats per la legislació europea, com el recompte de cèl·lules somàtiques, la bacteriologia o la presència de residus d'antibiòtics.

Les implicacions de la presència de residus d'antibiòtics en la llet, com resultat dels tractaments veterinaris, inclouen efectes negatius sobre la salut del consumidor com ara al·lèrgies o resistències bacterianes als antibiòtics i, també, problemes en la fabricació de productes lactis fermentats. Per a la detecció de residus d'antibiòtics en les mostres de llet hi ha diversos mètodes de garbellament disponibles sent els mètodes basats en la inhibició del creixement microbià i els assajos a base de receptors específics els més utilitzats en granges, indústries làcties i laboratoris de control. Estos mètodes de garbellament han sigut validats per al seu ús en llet crua de vaca, però la informació sobre els resultats d'estes proves en llet d'ovella i cabra és prou limitada.

L'objectiu d'este estudi ha sigut avaluar el funcionament d'alguns mètodes microbiològics i assajos ràpids de receptors per a la detecció d'antibiòtics en llet d'ovella i cabra segons la Decisió 657/2002/CE, a fi de determinar la seua idoneïtat per a controlar la presència de residus d'antibiòtics en la llet i establir l'estratègia analítica més convenient a Espanya.

La Capacitat de detecció (CCβ) dels mètodes microbiològics de garbellament, BRT MRL, Delvotest MCS SP-NT, Delvotest MCS Accelerator i Eclipse 100, va ser igual o inferior als límits màxims de residus (LMRs) establits per la legislació europea per a la major part d'antibiòtics betalactàmics estudiats i altres no betalactàmics com neomicina, tilosina, sulfadiazina i sulfadimetoxina. No obstant això, van ser menys sensibles per a detectar quinolones i tetraciclines als seus respectius nivells de seguretat. Quan es van analitzar mostres de llet individuals, els tests microbiològics van presentar una major incidència de resultats no conformes per a la llet d'ovella que va estar relacionada en tots els casos, amb un recompte elevat de cèl·lules somàtiques (RCS).

El Sistema microbiològic format per dos microplaques complementàries basades en la utilització de *Geobacillus stearothermophilus* var. *calidolactis* i *Bacillus subtilis*, respectivament, permet millorar els nivells de detecció en llet d'ovella respecte a l'ús d'un únic test comercial emprant *G. stearothermophilus*, al detectar algunes substàncies del grup de les quinolones i dels macròlids a concentracions més pròximes als seus respectius LMRs.

Els assajos ràpids de receptors (Charm MRL BLTET, Betastar Combo, SNAP Betalactam, SNAP Tetracycline i Twinsensor[BT]) van ser adequats per a detectar la major part de betalactàmics i tetraciclines a concentracions iguals o inferiors als LMRs (CCβ ≤ MRL). L'especificitat dels tests de receptors va ser elevada en tots els casos inclús quan es van analitzar mostres de llet individuals. Únicament el test Twinsensor[BT] va presentar resultats no conformes quan es van analitzar mostres lliures d'antibiòtics procedents d'animals individuals, especialment en les últimes setmanes de lactació. No es van observar reaccions encreuades amb la presència de substàncies pertanyents a altres famílies d'antibiòtics diferents de les de betalactàmics i tetraciclines en la llet. El conservant azidiol no va tindre cap efecte sobre la resposta dels tests de receptors. A més, no es van trobar diferències entre la interpretació visual i la instrumental dels resultats.

Tenint en compte la freqüència d'ús dels antibiòtics comunament empleats a Espanya i la sensibilitat dels mètodes a una concentració d'antibiòtic equivalent al LMR, es van calcular els ràtios totals de detecció en el garbellament. En general, l'ús d'un sol test permet detectar 62.8-82.4 % dels antibiòtics empleats. Per a la llet d'ovella, el rang total de detecció aconseguit amb els mètodes microbiològics va ser significativament major que l'aconseguit amb les proves ràpides de receptors. No obstant això, no es van trobar diferències significatives entre els dos tipus de tests quan es va analitzar llet de cabra. En ambdós tipus de llet, l'ús simultani de dos tests de garbellament amb diferent base analítica, va incrementar significativament el rang total de detecció aconseguint valors ≥ 90 % en alguns casos.

No obstant això, antibiòtics com ara enrofloxacina, marbofloxacina, espiramicina i estreptomicina que també s'utilitzen per a tractar la mamitis i altres malalties infeccioses del bestiar, no són detectades pels tests de garbellament avaluats. Per tant, la millora de l'estratègia analítica a través de l'aplicació periòdica de proves que detectaren la presència d'estes substàncies als nivells de seguretat establits seria recomanable.

Contents

Chapter 1

Introduction

1. ANTIBIOTIC RESIDUES IN MILK

1.1. General considerations

The use of veterinary drugs, especially antibiotics, in the treatment and prophylaxis of mastitis and other infectious diseases in dairy livestock is a widespread practice nowadays. However, the beneficial effects of antimicrobial therapy in lactating animals may counteract with the possible appearance of residues of these substances in milk, even several days after completion of the treatment. If, for some reason, milk containing antibiotics is introduced into the milk circuit, it may eventually contaminate milk stored in the refrigeration tank of the farm, in the tanker lorry or even in large industrial silos, causing what is known as "chained pollution" (Figure 1).
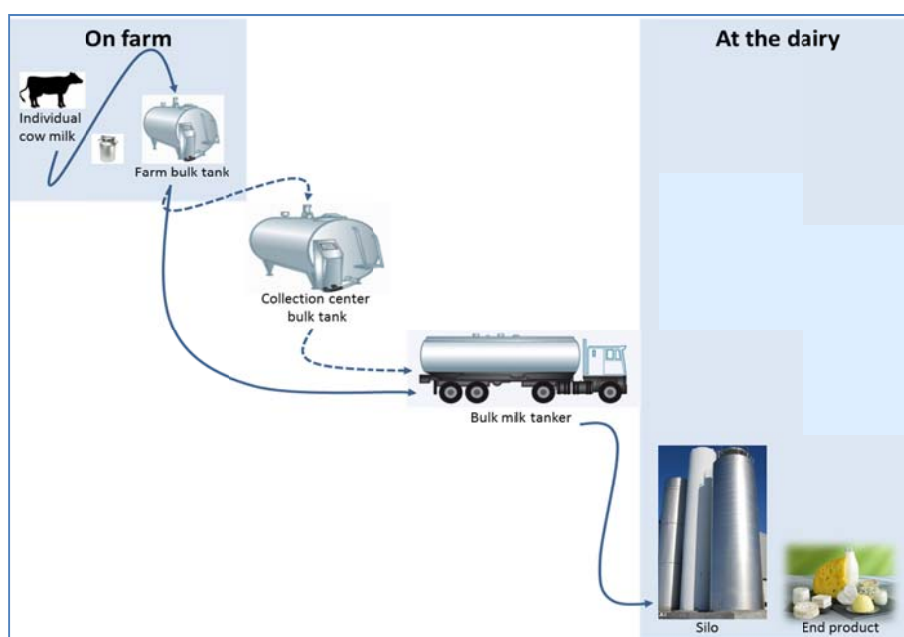
Figure 1. Diagram representing "chained pollution" in milk
Source: IDF (2013a)

There is an array of inherent factors to the application of antimicrobials that may influence the quantity and duration of the excretion period and therefore their presence in milk. Among them, the type of antibiotic, the dose, the route of administration, the influence of the expedient, and the health status of the udder (Anifantakis, 1982; Pedersoli et al., 1995; Stocker et al., 2009).

Research carried out to analyse the main causes of the presence of antibiotic residues in milk is very limited. In a study carried out in France by Fabre et al. (1995), 625 non-compliant results were detected during official controls on 1,018 farms along a one-year period. In 17 % of all cases, it was not possible to identify the cause of non-compliant results and, as shown in Figure 2, clinical mastitis treatments and dry cow

therapy were related to 64 and 24 %, respectively, causing positive results in the screening (n= 561), while the treatment of pathologies other than mammary was related to 11 % of the cases.
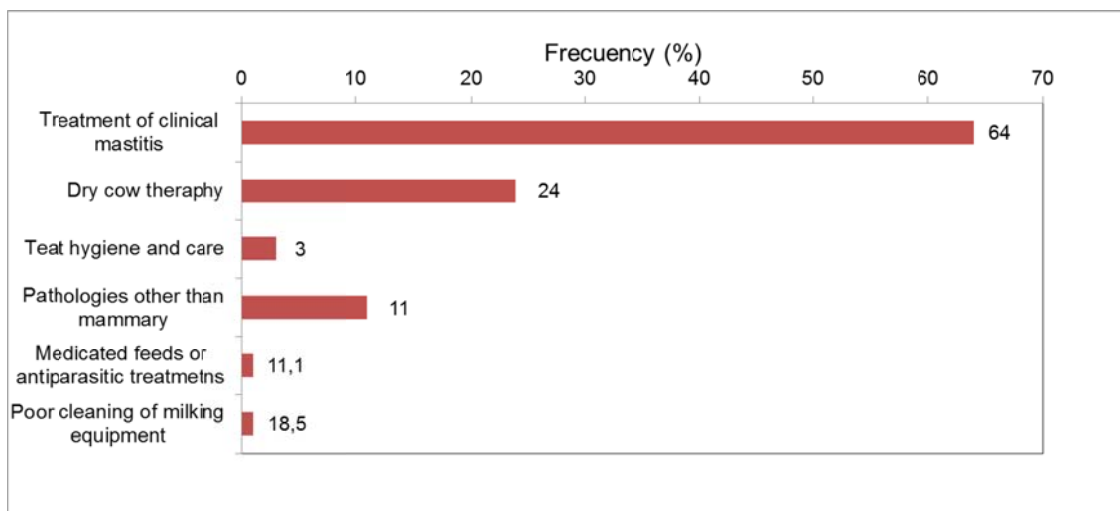
Figure 2. Main causes of the presence of inhibitors in milk
Source: Fabre et al. (1995)

Regarding clinical mastitis treatment (n=330), the main causes related to the presence of inhibitors in milk were accidental milking of treated cows (56 %) and the non-compliance of the dose and/or withdrawal period (38 %).

Also, in a study carried out in Spain by Sánchez et al. (2001) to investigate the causes of 175 positive results in the analysis of 95,000 cow milk samples from a dairy cooperative, mastitis treatment was implicated in all the cases detected, being mainly caused through negligence by the employees (30 %), the inadequate use of antibiotics (29 %) and the incorrect withdrawal of contaminated milk (22 %).

Considering these results, it can be concluded that the most causes of the presence of antibiotic residues in milk are mainly related to the irresponsible use of veterinary drugs in dairy livestock, clinical mastitis treatments being the most frequently cause incriminated. Thus, it is crucial to establish a code of good practices for antibiotic treatments in dairy animals to prevent residues in raw milk and reaching the food chain.

In this sense, the European Platform for the Responsible Use of Medicines in Animals (EPRUMA) in order to promote the responsible use of drugs in animals in the EU, as defined by Directive 2001/82/EC and amended by Directive 2004/28/EC, has published the document entitled "Best-practice framework for the use of antimicrobials in food-producing animals in the EU" (EPRUMA, 2008). This document provide guidelines for veterinarians and farmers alike to maintain efficacy and, at the same

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time, prevent and minimize adverse reactions provoked by the use of these substances.

More recently, the International Dairy Federation also published the "IDF Guide to Prudent Use of Antimicrobial Agents" (IDF, 2013b) in order to provide a generic framework to support the responsible use of antimicrobial agents on dairy farms. The guidelines recognize that a coordinated whole-of-supply chain approach is required to manage the food safety risks associated with modern food production.

1.2. Use of antibiotics in dairy sheep and goats

In the last decades, the farming of dairy sheep and goats has evolved towards more intensive production systems in which a high concentration of animals in a confined spaces is more common, increasing the risk of the occurrence of diseases and, thus, the use of veterinary medicinal products, especially antimicrobial drugs has therefore increased, too.
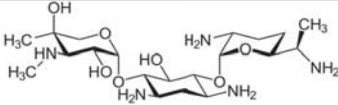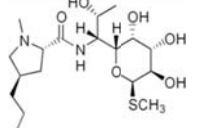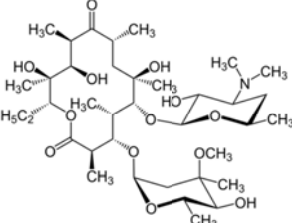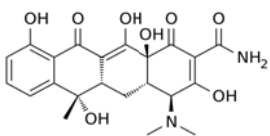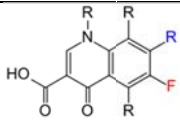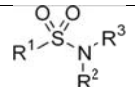
Table 1 summarizes the antimicrobial substances most commonly used to treat and prevent infectious diseases in veterinary medicine grouped according to their chemical structure, and including the main features of each of the groups of drugs considered.

Concerning the use of antibiotics in dairy sheep and goats, Berruga et al. (2008) conducted a study for the Ministerio de Medio Ambiente, Rural y Marino (MARM), currently Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA), on the most commonly drugs and treatments applied by veterinarians in Spain to study the possible causes of the presence of antimicrobial residues in milk from these species.

Mastitis is the main pathology requiring antimicrobial therapy in dairy sheep and goats. According to Berruga et al. (2008), a high percentage of veterinarians (72 % for sheep and 76.9 % for goats) usually treat clinical mastitis cases during the lactation period.

For the treatment of clinical mastitis in dairy sheep veterinarians primarily use beta-lactam drugs (56.8 %); macrolides being the second group of antimicrobials applied (Figure 3). Regarding lactating goats, mastitis is also treated using mainly beta-lactams (53.3 %) and macrolides (18.3 %). Compared with sheep, there is a greater tendency to use tetracyclines in goats (13.3 and 3.9 %, respectively) although their percentage of usage for this pathology is relatively low in comparison with the two other groups of antibiotics.

Table 1. Classification of the antimicrobial substances employed in veterinary medicine

Antimicrobial group	Features	Structure	Bacterial effect	Mechanism of action	Substances
Beta-lactams	• Natural antibiotics • Broad spectrum • beta-lactam ring		Bactericide	Cell wall synthesis inhibitors	• Penicillins: amoxicillin, ampicillin, benzylpenicillin, cloxacillin, etc. • Cephalosporins:cephalexin, ceftiofur, cefoperazone, cefquinome, etc.
Aminoglycosides	• Natural antibiotics • Amino-sugars and aminocyclitol ring		Bactericide	Protein synthesis inhibitors	gentamicin, kanamycin, neomycin, streptomycin, etc.
Lincosamides	• Natural antibiotics		Linked to concentration applied	Protein synthesis inhibitors	clindamycin, lincomicin, pirlimycin, etc.
Macrolides	• Natural antibiotics • Macrocyclic lactone ring		Bacteriostatic	Protein synthesis inhibitors	eritromycin, spiramycin, tilmicosin, tylosin, etc.
Tetracyclines	• Natural antibiotics • Broad-spectrum • Four hydrocarbon rings		Bacteriostatic	Protein synthesis inhibitors	chlortetracycline, oxitetracycline, tetracycline, doxycycline (semisynthetic derivate)
Quinolones	• Synthetic antimicrobials • Broad-spectrum		Bactericide	Acid nucleic synthesis inhibitors	enrofloxacin, marbofloxacin, norfloxacin, etc.
Sulphonamides	• Synthetic antimicrobials • Sulphonamide functional group		Bacteriostatic	Acid nucleic synthesis inhibitors	sulfadiazine, sulfadimethoxine, sulfametazine, sulfatiazol, etc.
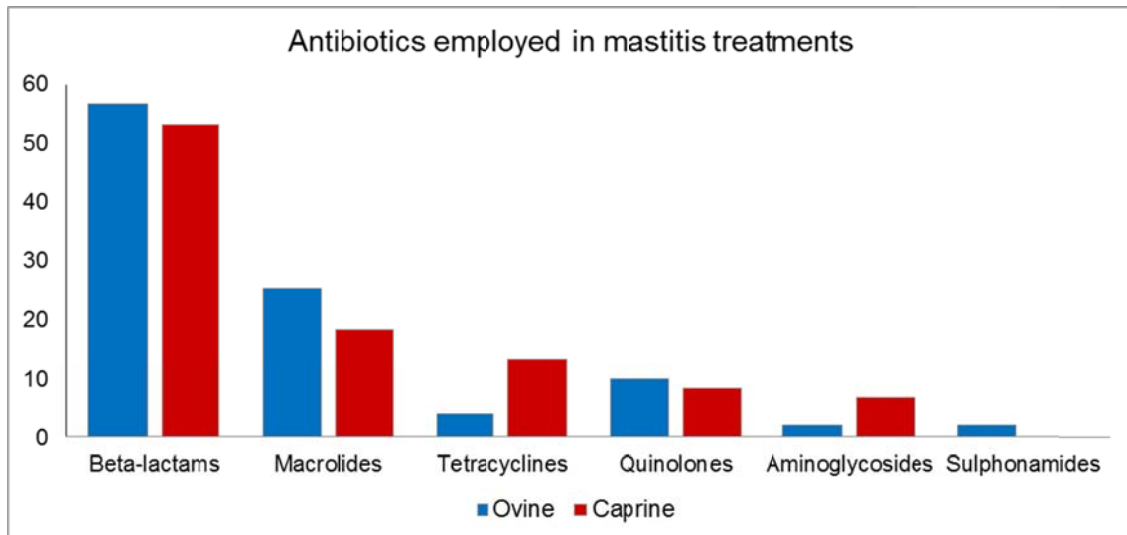
Figure 3. Frequency of use (%) of antimicrobial groups in the treatment of sheep and goats mastitis during lactation

Source: Berruga et al. (2008)

With regard to antibiotic dry-off therapy, most surveyed veterinarians indicated that they usually applied them (82 % for sheep and 73 % for goats) using mainly, just as in clinical mastitis treatments, beta-lactams and macrolides in either case (Figure 4).
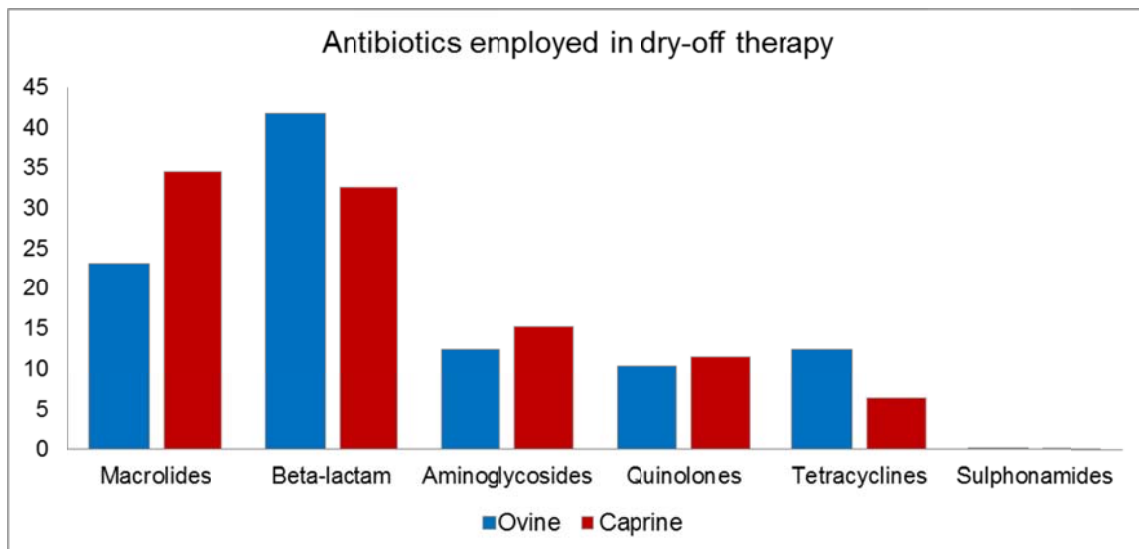
Figure 4. Frequency of use (%) of antimicrobials in dry-off therapy in sheep and goats

Source: Berruga et al. (2008)

In addition to intra-mammary infections, there are respiratory, reproductive and digestive diseases, among others, that require antimicrobial therapy in small dairy ruminants. To treat pathologies other than mastitis, tetracyclines are the antimicrobial group of choice by veterinarians both in dairy sheep (53 %) and goats (43.6 %); beta-

lactams (Berruga et al., 2008), being the second most commonly antimicrobial group applied (23.3 and 26.3 %, respectively).

An important aspect to be emphasized with respect to the use of antibiotics in dairy sheep and goats is that due to the low volume of business which represents milk production, in comparison with cow's milk, there is evidently a limited availability of drugs registered for these species, especially for goats (Veterindustria, 2012). This is forcing veterinarians to employ unregistered drugs to treat certain pathologies and, although legally the exceptional use of medicines is considered (Directive 2004/28/EC and Directive 2001/82/EC), implies a great responsibility for veterinarians as there is an increased risk of incidence of residues, given the lack of knowledge on the behaviour of these medications at pharmacokinetic and metabolic levels.

In addition, studies carried out in dairy sheep and goats (Molina et al., 2003a; Ferrini et al., 2010; Pergov et al., 2009) show that the withdrawal period of 7 days laid down in legislation for off-label treatments is not always sufficient to ensure the absence of antimicrobial residues in milk.

In this sense, Berruga et al. (2008) indicate that 67 % of veterinarians treating ovines and 76.9 % of veterinarians treating caprines admit the off-label use of antimicrobials, the application of veterinary drugs without record for the species being the most common practice (Table 2). The substances applied in off-label treatments include, in both species, cephalosporins, macrolides and quinolones.

Table 2. Exceptional use* of veterinary drugs in ovine and caprine

	Ovine (%)	Caprine (%)
Use of drugs registered for species other than sheep and goats	86.8	95.0
Administration through a pathway other than the one indicated	18.4	10
Use of drugs in doses other than those indicated	15.8	10
Applying a withdrawal period other than that recommended	5.2	7.5

*: Total number of veterinarians surveyed who admit the exceptional use of antimicrobial drugs
Source: Berruga et al. (2008)

Regarding the occurrence of antibiotic residues in sheep milk produced in Spain, sporadic studies have been carried out, in particular in the Castile and León, and Castile-La Mancha regions, using microbial inhibitor tests in all cases (Table 3).

Table 3. Occurrence of antibiotic residues in sheep milk produced in Spain

Spanish Region	Year	Microbial test	Positive results	Groups of drug residues identified	Reference
Castile-La Mancha	1999	BRT	6.1 %	Beta-lactams 4 %	Althaus et al. (2007)
				Sulphonamides 1.2 %	
Castile and León	-	Eclipse 100	11 %	-	Esnal et al. (2002)
		MMS[1] (5 plates)	22.7 %	Beta-lactams	
				Macrolides	
				Tetracyclines	
Castile-La Mancha	2002-2003	Delvotest SP	1.3 %	Beta-lactams 29.8 %	Yamaki et al. (2004)
		Eclipse 100ov	0.9 %	Beta-lactams 25 %	Yamaki et al. (2006)

[1]MMS: Microbiological multiplate system

More recent reports have shown that the scenario has considerably improved and, currently, the occurrence in the main Spanish sheep milk-producing regions indicate incidences ≤ 0.15 % (Brusa and Safigueroa, 2005; Gonzalo et al., 2013). These low values are similar to those presently seen in cow milk, which verifies the improvement of the quality of sheep milk in recent years with regard to the presence of inhibitors.

Studies on the incidence of inhibitor residues in goat's milk are very scarce. In a study conducted by Marco et al. (2001) 12.7 % positive samples were obtained in the Murcia region, one of the main producers of goat's milk in Spain. In goat's milk, just as in sheep milk, the occurrence of positive results has been reduced in recent years. Gonzalo et al. (2012), in a study performed in the Castile and León region, indicate a decrease in the occurrence of antibiotic residues in bulk milk samples from goats from 0.3 % in the year 2005 to less than 0.001 % in 2011 evidencing the improvements made in the sector.

1.3. Effect of the presence of antibiotic residues in milk

The consumption of milk containing residues of antibiotics can produce harmful effects on human health, causing transient disturbances in the intestinal flora and allergic reactions which can, in extreme cases, lead to anaphylaxis (Tollefson et al.,

2004; Demoly and Romano, 2005; Sanders et al., 2011). There is also the concern that the presence of antibiotics in foodstuff may be responsible for the development of bioresistance (Swarchz et al., 2001; Philips et al., 2004; Oliver et al., 2011). In this sense, during the last decade, the World Health Organization and the Organization World Animal Health together with the United Nations Organization for Food and Agriculture and the Codex Alimentarius Commission have addressed the potential risk posed by the use of antimicrobials for the treatment of diseases of animals intended for food production that appear and extend organisms resistant to antimicrobial agents.

In addition, some of these substances are not destroyed with heat treatments commonly applied to milk in the dairy industry in order to reduce the microbial load and eliminate pathogens and enzymes, which can reach the consumer even after having undergone these treatments (Zorraquino et al., 2008, 2009, 2011; Roca et al., 2010). Neither do the manufacturing processes of yoghurt and cheese seem to influence the concentration and activity of some antibiotic residues (Grundwald and Petz, 2003; Adetunji, 2011).

On the other hand, from a technological point of view, the presence of antimicrobial residues in milk may inhibit the bacterial processes required for the elaboration of fermented products such as cheeses and yoghurt (Cogan, 1972; Packham et al., 2001; Berruga et al., 2007). Technological damage produced by residues depends on the type of antibiotics, its concentration in the milk and the type of product manufactured (Mäyrä-Mäkinen, 1995). This is a very important aspect when considering that milk from sheep and goats is primarily intended for the manufacture of milk products especially cheese and yoghurt.

Many regions of the Mediterranean basin are characterized by a great tradition of sheep and goat's milk production that is intended for the manufacture of pure cheese of these species, many of them under the protected designation of origin (PDO) and other brands of quality of international recognition. In Spain there are many PDOs for sheep (Manchego, Idiazábal, Roncal, Zamorano, La Serena, Torta del Casar) and goat's (Ibores, Murcia, Palmero, Majorero) cheeses representing significant economic value for the regions of production.

Another important aspect to consider is the possible economic impact of the presence of antibiotics in milk for the farmer because it can lead to a ban by the competent authorities, if the marketing of raw milk is considered "unfit for human consumption". The possible restriction of the commercialization of the contaminated

milk together with the storage costs and subsequent elimination are the responsibility of the farmer and, therefore, represent major economic losses.

It should also be considered that significant amounts of antibiotics administered to animals are not metabolized and eliminated by milk, urine and/or faeces (Kemper, 2008), causing them to contaminate the top coat of the soil where they can accumulate or seep into the groundwater (Martinez-Carballo et al., 2007) and can affect the microflora, the microfauna and the groundwater quality, having serious environmental implications (Figure 5).
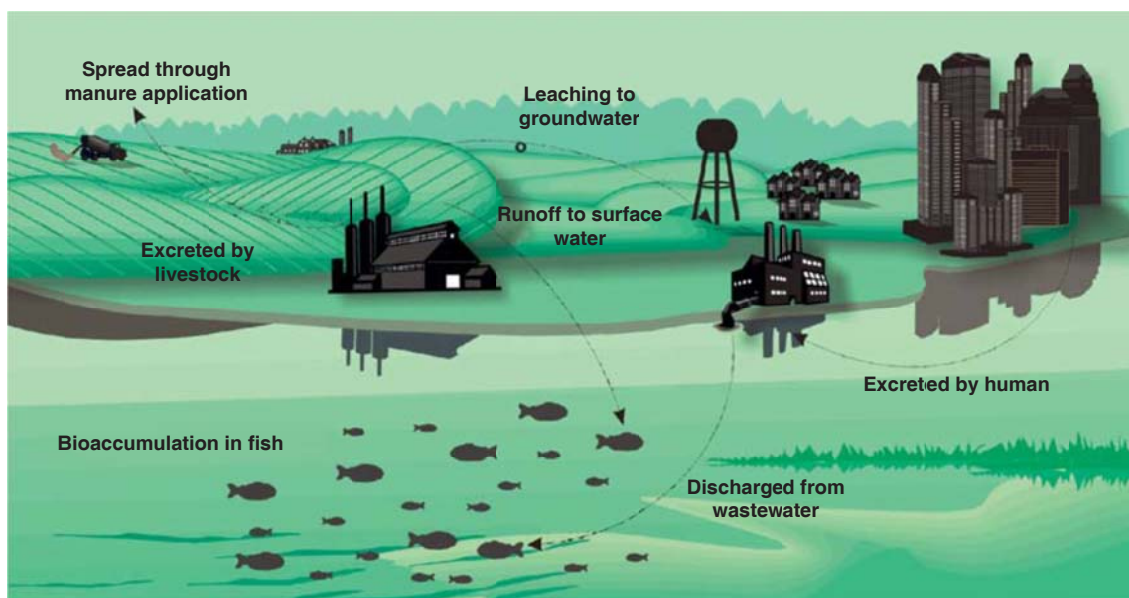
Figure 5. Sources and transport of human and veterinary antibiotics in the environment
Source: Mojica and Aga (2011)

2. LEGISLATIVE ASPECTS. SAFETY LIMITS

2.1. General information

In the European Union, the control of the presence of antibiotic residues in milk and other products of animal origin is regulated by Council Directive 96/23/EC, requiring European member states to monitor antibiotic residues and other veterinary medicinal products and contaminants within a national residue monitoring plan. The sampling levels and frequencies for the monitoring of certain substances and residues in milk and other animal products provided for by Council Directive 96/23/EC are fixed by Commission Decision 97/747/EC.

The principal objective of this legislation is to detect the use of illegal substances in animal production and the misuse of authorized veterinary drugs to ensure the implementation of appropriate actions to minimize the presence of residues in food of animal origin. To monitor the correct operation of the control plans the EU

has designated four reference laboratories for the detection of residues of certain substances, as well as the corresponding national laboratories designated by each member state.

After passing Regulation (EC) Nº 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and defining procedures in matters of food safety, the EU began the publication of an extensive legislative framework in the field of food hygiene, which constitutes the general, basic and common principles for the production and marketing of all foodstuffs according to hygienic standards, and in particular, in the field of the production of raw milk from sheep and goats.

Also, the responsibility of other operators of the food chain in the production of safe food was reaffirmed. Regulations (EC) Nº 852/2004 on the hygiene of foodstuffs, and (EC) Nº 853/2004 lay down specific rules of hygiene of foodstuffs of animal origin, in particular, general and specific standards of hygiene for the production of various food including raw milk of sheep and goat. To ensure compliance with these hygienic rules the European Union also published Regulation (EC) Nº 854/2004 setting up specific rules for the organisation of official controls on products of animal origin intended for human consumption.

2.2. Maximum Residue Limits

In order to protect public health, maximum residue limits (MRLs) of pharmacologically active substances in foodstuffs of animal origin have been established in accordance with procedures provided by Regulation (EC) Nº 470/2009 repealing Council Regulation (EEC) Nº 2377/90 and amending Directive 2001/82/EC and Regulation (EC) Nº 726/2004. This Regulation defines the MRL as the maximum concentration of a residue of a pharmacologically active substance which may be permitted in food of animal origin.

The MRLs established apply the concept of acceptable daily intake (ADI) which corresponds to the amount of a residue that a human being may ingest on a daily basis with food along their lifetime without suffering any type of harm. The ADI is used together with data on pharmacokinetics, depletion of residues and the knowledge concerning the marker residue and target tissues to establish the MRLs, both for the pure substance, and its metabolites, in food of animal origin (muscle, fat, liver, kidney, milk, honey and eggs). The calculation of the MRL depends on the animal tissue and species considered and the amounts of each of them that can be ingested by consumers on average.

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For pharmacologically active substances prohibited or not regulated by the EU, Regulation (EC) Nº 470/2009 has also set up a procedure to establish "the reference values for purposes of intervention", defined as the lowest concentration of a residue that can be detected and confirmed by an official laboratory, previously known as minimum required performance limits (MRPLs).

Commission Regulation (EU) Nº 37/2010 lists the MRLs established for different substances in foodstuff of animal origin, classified into two groups: allowed substances and prohibited substances. Some antimicrobials, grouped by families, for which MRLs in milk from different species have been established, are shown in Table 3.

Tabla 3. Maximum residue limits (MRLs) established in the EU for antimicrobial substances in milk from different species.

Substance	MRL (μg/kg)	Type of milk	Substance	MRL (μg/kg)	Type of milk
Beta-lactams			*Aminoglycosides*		
Amoxicillin	4	All[1]	Gentamicin	100	Bovine
Ampicillin	4	All	Kanamycin	150	All
Benzylpenicillin	4	All	Neomycin	1,500	All
Cloxacillin	30	All	Spectinomycin	200	All
Dicloxacillin	30	All	Streptomycin	200	All rum
Nafcillin	30	All rum[2]			
Oxacillin	30	All	*Quinolones*		
Cefacetrile	125	Bovine	Danofloxacin	30	BOC
Cefalexin	100	Bovine	Enrofloxacin	100	BOC
Cefalonium	20	Bovine	Flumequine	50	BOC
Cefazoline	50	BOC[3]	Marbofloxacin	75	Bovine
Cefoperazone	50	Bovine			
Cefquinome	20	Bovine	*Sulphonamides*		
Ceftiofur	100	All	Sulfadiazine	100	Bovine
Cephapirin	60	Bovine	Sulfadimethoxine	100	Bovine
Penethamate	4	All	Sulfadoxine	100	Bovine
			Sulfanilamide	100	Bovine
Tetracyclines			Sulfametazine	100	Bovine
Chlortetracycline	100	All	Sulfatiazol	100	Bovine
Oxitetracycline	100	All			
Tetracycline	100	All	*Others*		
			Bacitracin	100	Bovine
Macrolides			Baquiloprim	30	Bovine
Erytrhomycin	40	All	Clavulanic acid	200	Bovine
Spiramycin	200	Bovine	Colistin	50	All
Tylosin	50	All	Novobiocin	50	Bovine
			Rifaximin	60	Bovine
Lincosamides			Thiamphenicol	50	All
Lincomycin	150	All			
Pirlimycin	100	Bovine			

[1]All= all food producing species; [2]All rum= All ruminants; [3]BOC= Bovine, ovine, caprine
Source: Regulation (EU) Nº 37/2010

2.3. Quality control and traceability of the milk in Spain

Regulation (EC) Nº 178/2002 directed at food companies establishes the need to implement systems that allow ensuring the traceability of foodstuffs at all stages of production, processing and distribution. In order to apply Community legislation with regard to the traceability of raw milk, the Ministerio de Agricultura, Pesca y Alimentación, currently MAGRAMA, issued Real Decreto 217/2004, identifying and registering agencies, establishments and entities involved in the dairy sector as well as the registration of the movements of raw cow milk.

This Real Decreto created the tool which allows the traceability of the raw cow milk in Spain, i.e. the "Letra Q database" module (LEche, TRAzabilidad, Qualidad), which is a software application integrated into the information system "Letra Q", registering all the agencies and containers used within the dairy sector. The dairy centres responsible record all the container movements that occur from the obtention of raw milk in cattle, until reaching a dairy centre for processing in the "Letra Q database". Figure 6 shows a diagram of the system to ensure the traceability of milk, stipulated by Real Decreto 217/2004.
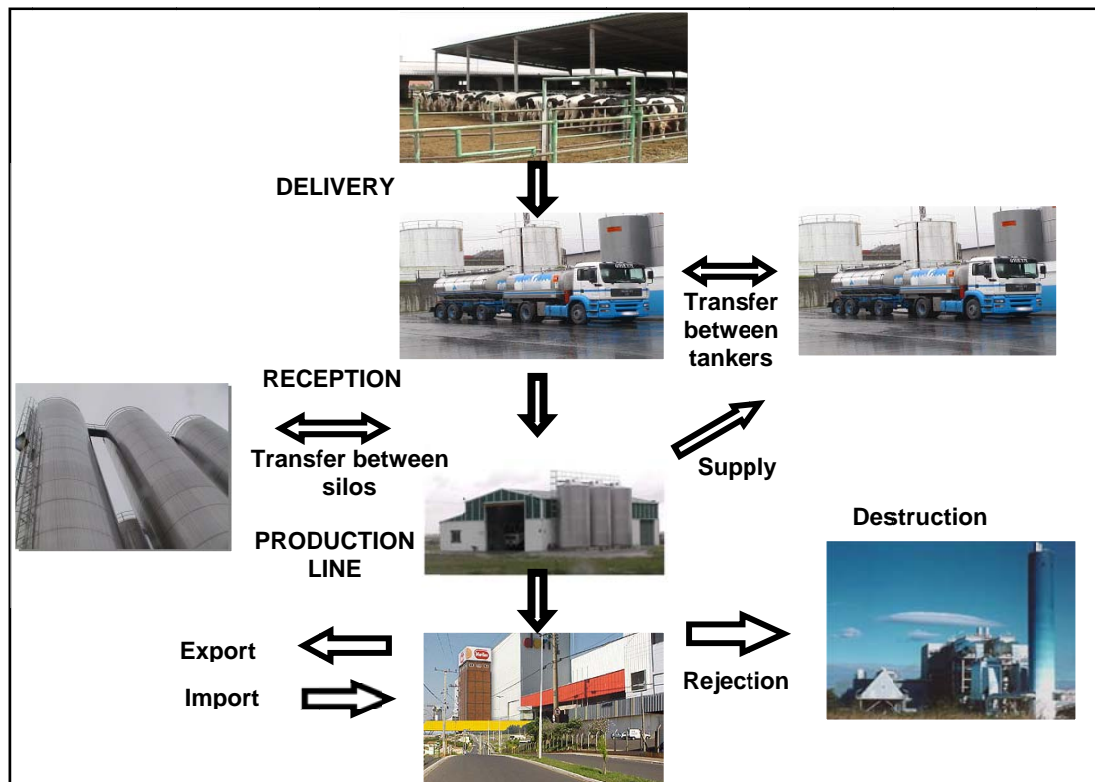
Figure 6. Diagram of the different stages involved in the system of management of the traceability of the raw milk

Source: MAPYA (2006)

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In addition, in order to ensure compliance with the hygienic requirements referred to in regulations (EC) Nº 852 and 853/2004 ensuring that official controls of the presence of residues in animal products intended for human consumption covered by regulations (EC) Nº 854 and 882/2004, Real Decreto 1728/2007 establishes the basic rules of control to be met by operators in the dairy sector and amending Real Decreto 217/2004, which regulates the identification and registration of agencies, establishments and entities involved in the dairy sector, and recording the movements of milk.

Subsequently, Real Decreto 752/2011, which allows the development of Community regulations at a national level with regard to raw sheep and goat's milk production, was published establishing mandatory minimum controls to be performed by food-producing agencies, as the harmonisation of the conditions required from laboratories for the analysis of raw milk from sheep and goats, and the homogenous activity thereof before sampling, analysis and communication to the competent organ. Additionally, the obligation to transmit the results generated in the implementation of the checks carried out to the "Letra Q database" was extended to the dairy sheep and goat sector.

Monitoring the presence of antimicrobials in raw milk from sheep and goats is included among the tests to be carried out in farms before loading milk onto the collection tanker, if there is suspicion or certainty of the presence of drug residues in milk. Screening for antibiotic residues *in situ* prior to the discharge of milk into the storage silos is mandatory in the dairy centres. In both cases, Real Decreto 752/2011 requires the use of methods capable of detecting, at least, beta-lactam antibiotics. The actions to be taken according to the result of the test for the detection of antibiotics *in situ* are outlined in Figure 7.

As for the screening test of antibiotic residues that should be performed in control laboratories, annex IV of this regulation establishes that, for all milk samples received methods able to detect, at least, beta-lactam residues must be employed.

All official laboratories shall communicate to the "letra Q database", the results obtained for the analysis of samples from self-control in farms before loading milk, and the results corresponding to the samples from the tankers prior to their discharge in storage silos, at dairies. The "Letra Q database" will generate alarms or warnings to the competent authorities of the autonomous communities to communicate breaches in somatic cell counts and bacteriology monthly and positive results to the test of

antibiotic residues daily. In case of defaults, especially in the test of antibiotic residues, immobilization of the milk and to its subsequent destruction will be carried out.
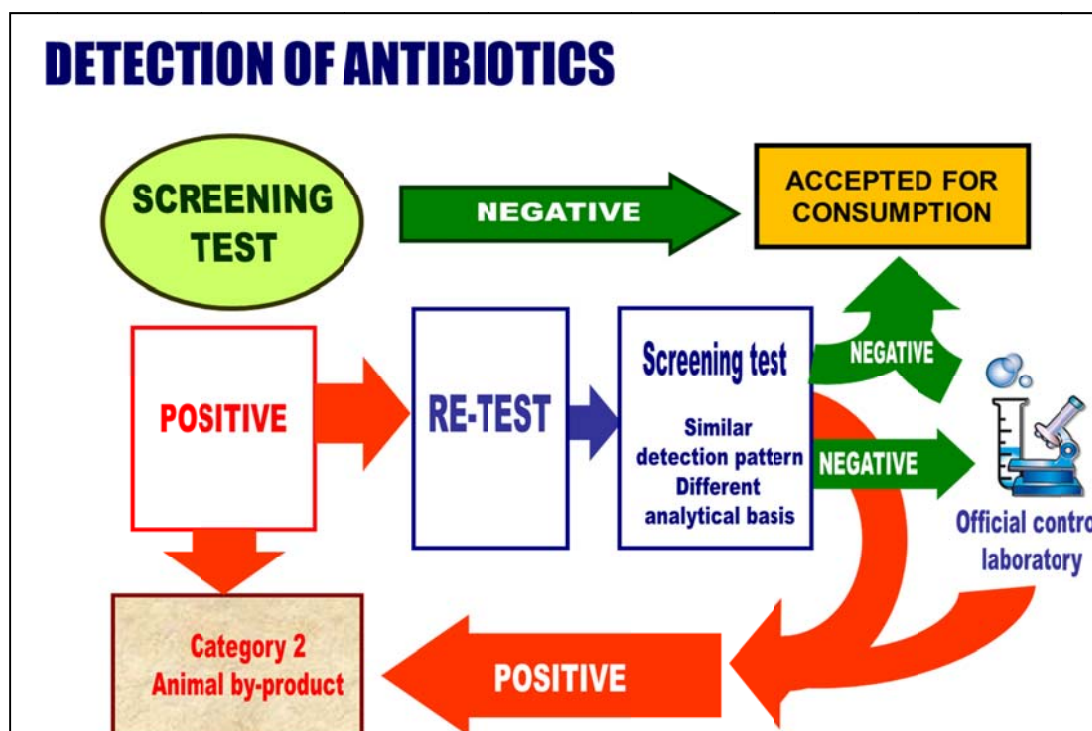
Figure 7. Diagram on the testing performance for the detection of antibiotic residues in farms and dairies

Source: MAPYA (2007)

3. METHODS FOR THE DETECTION OF ANTIBIOTICS IN MILK

3.1. Classification of methods for the detection of antibiotics

The first methods for the detection of antibiotic residues in milk were used around the 1950s and basically consisted of evidence of inhibition of microbial growth (Bishop and White, 1984). Since then, many of the performance characteristics of these methods as the rapidity of response, accuracy and sensitivity have been improved and also, several screening methods based on immunological techniques, microbial, or protein receptors have been developed, that greatly reduce trial times.

Of the new technologies, electrochemical and optical immunosensors, flow cytometric immunoassays and biochip array technology applications for residue analysis are presently under evaluation (Conzuelo et al., 2012; Suárez-Pantaleón et al., 2014) offering a very promising future in the field of the detection of residues in food.

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The detection of residues of antibiotics in milk is a complex issue and to successfully address this task it is necessary to use an analytical strategy that adequately combines the methodologies currently available in order to detect the greatest number of substances at the least possible economic cost.

Thus, to ensure the safety of milk and dairy products in the EU an integrated system of control (Figure 8) with shared responsibilities for farmers, processors and food inspection is employed, in which a primary screening to detect samples potentially non-compliants and a phase of confirmation to verify the presence of residues above the limits legally established can be distinguished.
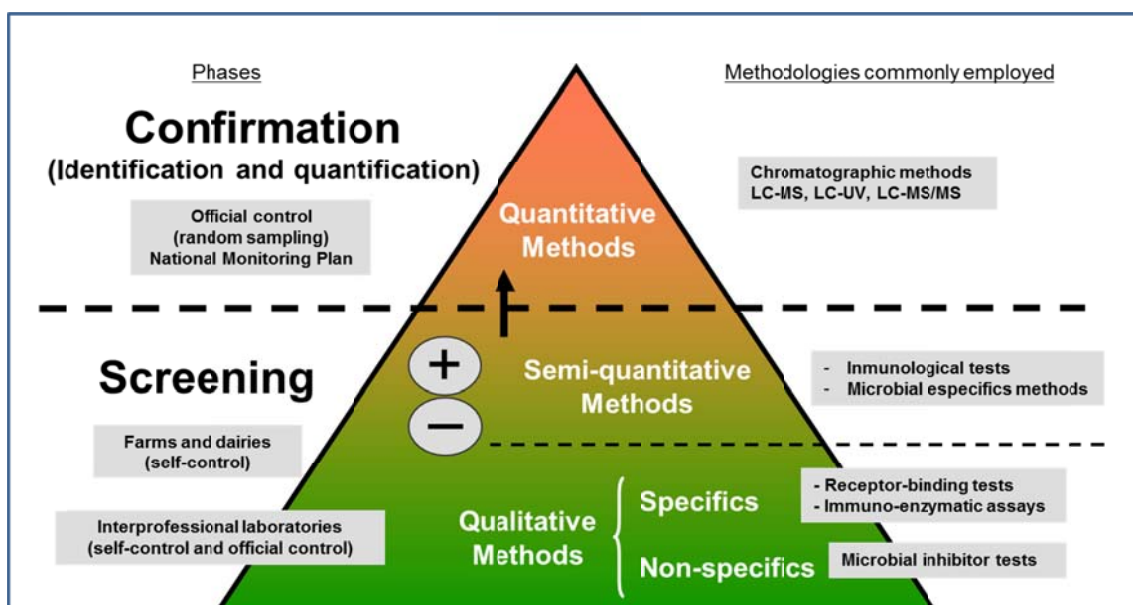
Figure 8. Pyramidal structure for a cost-effective monitoring for antimicrobials in milk

As a result of advances in analytical chemistry since the adoption of Directive 96/23/EC, the concept of routine methods and reference methods has been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established. Consequently, Commission Decision 657/2002/EC provides rules for the analytical methods to be used in the testing of official samples and specifies common criteria for the interpretation of analytical results of official control laboratories for such samples.

Commission Decision 657/2002/EC classifies analytical methods to detect residues in milk according to their operating characteristics into qualitative methods, which are those that identify a substance on the basis of its chemical, biological or physical properties, and quantitative methods, which determine the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units.

Qualitative methods are used for screening antibiotic residues in milk, microbial inhibitor tests being the most frequently used in control laboratories to detect the presence/absence of antibiotic residues in milk above the safety limits legally established. Screening methods also include qualitative specific methodologies that allow a preliminary confirmation of residues.

Microbial screening methods are based primarily on evidence of inhibition of the growth of a specific organism, using for the detection of this inhibition, different systems as indicators of pH, redox, bioluminescence. These methods primarily take advantage of the ability of bacteria to produce acid, reduce dyes or produce inhibition halos in a culture medium, so that the result can be interpreted visually.

The microorganisms used in these methods include among others: *Geobacillus stearothermophilus* var. *calidolactis* (Carlsson and Björck, 1987), *Bacillus cereus* (Suhren et al., 1993), *Bacillus subtilis* (Aureli et al., 1996) and *Streptococcus thermophilus* (Mourot and Loussouron, 1981). During the incubation period milk spread through the culture medium and if it contains sufficient quantity of antimicrobial substances the microorganism growth will be reduced or inhibited.

Currently, commercial microbiological tests most frequently applied in the screening of antibiotics in milk use *Geobacillus steraothermophilus* var. *calidolactis* as microorganism-test as it is very sensitive to beta-lactam antibiotics. Thus, the BRT (Analytik in Milch Produktions-und Vertriebs-GmbH, Germany), Delvotest (DSM Food Specialties, the Netherlands), and Eclipse 100 (Zeulab, Spain) tests, some of the most commonly used tests in Spain, having detection capabilities near their respective MRLs for a great number of substances belonging to this group of antimicrobials. However, the same does not applied to other groups of antibiotics such as tetracyclines, aminoglycosides, quinolones and sulphonamides (Le Bréton et al., 2007; Stead et al., 2008).

On the other hand, among the specific qualitative methods used in the screening stage, there are currently commercially available different types of enzymatic, immunological and receptor-binding assays, allowing detection of antibiotic residues in milk in a specific and usually in a much faster manner.

Receptor-binding assays using lateral flow technology are the most usually employed for screening antibiotics in farms and dairies as they are easy to use and rapid response (<10 min). Some of the most commonly used in Spain are the ROSA Charm (Charm Sciences Inc., MA, USA), Betastar (Neogen Corporation, MI, USA), SNAP (IDEXX Laboratories, ME, USA), and Twinsensor[BT] (Unisensor, Belgium) tests,

having versions able to detect individual or simultaneously, antibiotics belonging to beta-lactam and tetracycline groups.

These tests are based on the union of the antibiotic present in milk to proteic receptors, conjugated to an enzym, that are specific for an antibiotic or a certain group of antibiotics. All tests permit the visual interpretation of the test results by comparison of coloured lines or spots appearing as a result of the interaction of the analyte and the receptors contained in the test, but they also offer the possibility of reading results using automatic equipment which provide readings more objectively.

As for the confirmation phase, physico-chemical methods able to provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest are used. Numerous methodologies for the quantitative analysis of antimicrobial residues in milk have been developed. In general, physico-chemical methods for the confirmation of antimicrobials are based on the chromatographic separation of residues, especially Liquid Chromatography (LC), followed by spectroscopic quantification, such as UV, fluorescence or mass spectrometry (MS).

Despite the associated costs, mass spectrometric methods have been used more and more in last years for very selective and specific multi-compound detection; the LC-MS analytical method being most employed in milk and other foods (Cháfer–Pericás et al., 2010).

Table 4 summarizes the various types of antibiotic testing methods: principle, typical techniques involved, specificity and precision as well as practical details on equipment, cost, time and operator skills required.

3.2. Criteria for the validation of methods for the detection of antibiotics

As shown previously, the different control purposes require different categories of methods. Commission Decision 2002/65/CE establish the performance characteristics that should be verified for the validation of the analytical methods used for the detection of antibiotic residues in milk (Table 5).

Screening methods are used to detect the presence of a substance or class of substances at the level of interest. In agreement with Commission Decision 2002/657/EC they are characterised by high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are also specifically designed to avoid false compliant results. The performance characteristics that should be evaluated for their validation are explained as follows.

Table 4. Antibiotic testing methods: principles and results delivered

	Screening					Confirmation
Result	Qualitative = positive/negative		Semi-quantitative with an estimated concentration		Quantitative with an accurate concentration	Confirms antibiotic identity and accurate concentration
Detection	Biological	Biochemical	Biological	Biochemical	Physico-chemical	Physico-chemical
Principle	Detects cellular metabolic responses to analytes	Detects molecular interactions between antibiotics and ligands (antibody or receptor protein)	Detects cellular metabolic responses to analytes	Detects molecular interactions between antibiotics and ligands (antibody or receptor protein)	Separation of individual antibiotics and physical detection	Separation of individual antibiotics and physical detection
Typical techniques	Bacterial growth inhibition	Immunoassay	Bacterial growth inhibition	Immunoassay	Chromatography + spectrometry	Chromatography + mass spectrometry
Methods	Incubation with bacteria in solution or on plates or ampoules	Lateral flow, ELISA, biochip or Radioimmunoassay	Plate test / inhibition zone	Specific ELISA	LC-UV, LC-FL, LC-ECD, LC-MS, GC-FID	LC-MS/MS or LC-HRMS
Interpretation	Visual or colorimetric (pH or redox indicator) Readers available	Visual or colorimetric Readers available	Visual based on size of inhibition zone Quantitative estimation possible only if known substance	Colorimetric (labelling) with calibration curve Micro plate reader	UV or FL-spectrometry with calibration curve	Mass spectrometry with calibration curve
Analysis time Precision	1-3,5 h -	2-10 min to 3 h -	Several hours Low	2-4 h Medium	1-2 h High	1-2 h High
Specificity	Not specific Broad spectrum, several antibiotics and one or several families	Specific for one or several antibiotics or families	Not specific Antibiotic families	Specific for one single antibiotic	Identification determination of individual antibiotics	Spectrometric identification and determination of individual antibiotics
Antibiotics range analysed simultaneously	Large range	Single antibiotic or one or more families	Medium to large range	Single antibiotic	Small to medium range	Medium to large range
Cost	Cheap	Cheap/medium	Cheap	Medium	Medium/expensive	Expensive
Sample preparation	None or simple	None or simple	Medium	Simple to complex	Complex	Complex
Equipment/complexity	Simple	Simple or medium	Simple	Medium	Medium	High
User skills / training	Low	Low/Medium	Low	Medium	Medium/high	High
Typical application level	From farm to dairy	From farm to dairy	Collection Center to dairy	Dairy silo	Dairy silo	Finished product

Source: IDF (2013a)

Table 5. Classification of analytical methods by the performance characteristics that have to be determined

		Detection limit (CCß)	Decision limit (CCα)	Trueness/ recovery	Precision	Selectivity/ specificity	Applicability/ ruggedness/ stability
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+

S= screening methods; C= confirmatory methods; += determination is mandatory

Source: Commission Decision 2002/657/EC

Detection capability (CCβ)

Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1 – β$. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of $1 – β$ (Commission Decision 2002/657/EC).

For the calculation of the CCβ of a microbial test or receptor-binding assay for screening antibiotic residues in milk, the International Dairy Federation (ISO/IDF 2002 and 2003, respectively) recommends a calculation procedure that includes the use of different concentrations of antibiotic, in order to build a dose-response curve (Figure 9) from the positive frequencies for each concentration assessed, making a total of 10-20 replicates if the interpretation of the test results is made visually, and 3-5 if it is photometric.

Test concentrations must include a negative control (antibiotic-free milk sample), a concentration at least, 1.5 to 2 times higher than the concentration that is expected to be positive and a concentration equivalent to the maximum residue limit (MRL), calculating the limit of detection (CCβ) as the concentration which corresponds to the intersection of the dose-response curve with the line that represents the 95 of positive results (Figure 9).
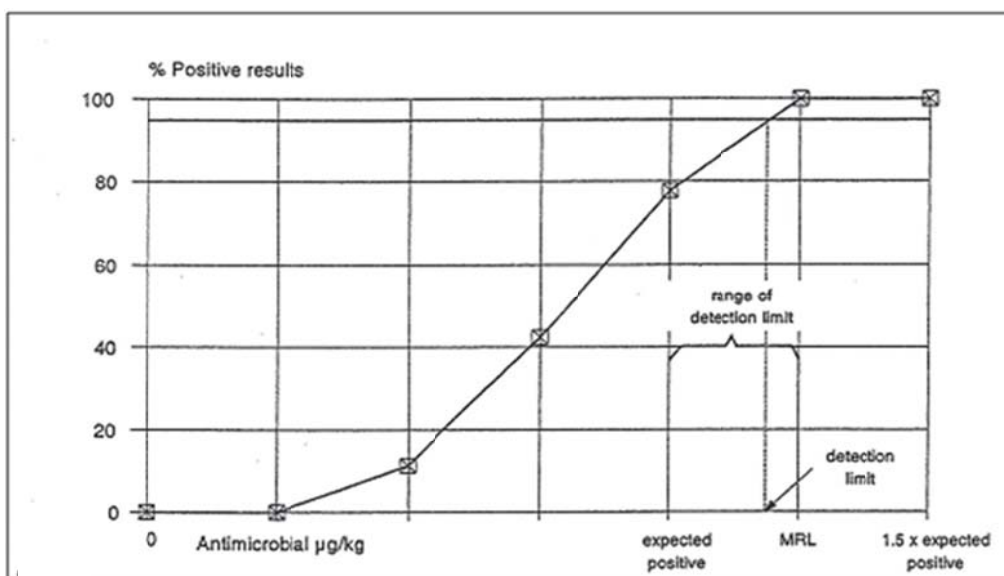
Figure 9. Dose-response curve model for the calculation of the detection limit of the screening methods
Source: ISO/IDF (2002, 2003)

More recently, Community Reference Laboratories for residues have published a document entitled "Guidelines for the validation of screening methods for residues of veterinary medicines" (CRLs, 2010). This guideline document supplements Commission Decision 2002/657/EC, and defines CCβ as the concentration at which only ≤ 5% false compliant results remain.

For authorized analytes, the concentration at which a screening test categorizes the sample as screen positive (potentially non-compliant), which triggers a confirmatory test, is called screening target concentration (STC) and it must be at or below EU-MRL. If the STC is set at half EU-MRL, the occurrence of one or no false-compliant results following the analysis of at least 20 screen positive control samples is sufficient to demonstrate that CCβ is below EU-MRL and below or equal to 50 % of EU-MRL. If STC is set between 50 % and 90 % of EU-MRL, at least 40 screen positive control samples with no more than 2 false-compliant results will be sufficient to demonstrate that CCβ is below EU-MRL. If STC approaches EU-MRL (< 10 % of EU-MRL) a maximum of 60 replicates with no more than 3 false-compliant results is required to demonstrate that CCβ is fit for this purpose.

Specificity

Specificity means the ability of a method to distinguish between the analyte being measured and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to the class of compound or matrix properties.

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This parameter is, therefore, associated with the presence of false-positive results and is of great interest to evaluate the analytical capacity of a test. For its determination a large number of milk samples from animals not treated with veterinary medicinal products should be analysed (ISO/IDF, 2002 and 2003).

In specific screening tests such as receptor-binding assays, specificity is related to positive results obtained when found in milk substances belonging to other groups of antimicrobials that could interfere with the test response (cross reactivity). For its calculation large amounts of potentially interfering substances are added into milk samples, and analysed in search of non-compliant results.

Therefore, a good screening method must be designed to present high values of specificity, i.e. a low percentage of false-positives.

Ruggedness

Ruggedness means the susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, and temperature) any variation which could affect the analytical result should be indicated.

Table 6 summarizes the use and the limitations of the qualitative screening tests most frequently used at present to detect antibiotic residues in milk. As seen in Table 6 all screening tests should be validated according to official validation guidelines before their routine use in practice. In fact, in some countries such as France or Belgium, validation of analytical screening tests to detect residues of antibiotics in accredited official laboratories is required to be approved for use in official milk quality programmes.

3.3. Use of screening tests in sheep and goat's milk

Screening methods currently available for the detection of antibiotic residues in milk have been developed and optimized for the use in cow's milk but there are no specific methods to be used in sheep and goat's milk. However, the different characteristics of milk from these species with regard to cow milk may sometimes lead to incorrect results using these control methods.

Table 6. Antibiotic qualitative screening tests: use and limitations

Type of test	All screening tests: Immuno- or cell based	Microbial growth inhibition in solution/in agar	Rapid tests : lateral flow device "dipstick" or "strip test"	ELISA
Antibiotic coverage & fitness for purpose	Make sure test detects antibiotics used where the milk is produced. Check sensitivity/ specificity data provided by suppliers vs. required local regulatory limits.	Reasonable to good detection of ß-lactams. Some antibiotics not detected at MRL (e.g. some amino-glycosides, tetracyclines and most sulfonamides)	Several types: mono- and multifamily tests. One line per family Sometimes not all compounds of a family are detected at or below MRL	Generic : targets one antibiotic family - the response of individual compounds varies and is expressed as % cross-reactivity Specific: targets selectively single drug e.g. banned antibiotics
Critical factors	Test storage (cold chain), Incubation time or temperature	Operator variability in case of visual reading	Operator variability in case of visual reading	Sample preparation: test recoveries with fortified or incurred samples (reference samples)
Strength		Broad spectrum	Very quick	Delivers estimated concentration
Controls/ precautions	Follow supplier instructions Positive & negative controls are highly recommended to test operators ability to execute the test properly	Carefully monitor expiry dates. Detection capability may change over shelf life Neg control: blank milk Pos control: provided by supplier or prepared by spiking standard into blank milk	 Neg control: blank milk Pos control: provided by supplier or prepared by spiking standard into blank milk	 Positive antibiotic standard solution or solutions for calibration curve included in the test kit
Interferences leading to false negatives	Test does not have the right selectivity/sensitivity profile (see fitness for purpose) Betalactamase can decrease beta-lactam concentrations*	Acidic pH (sour milk) can give false negatives with tests using pH indicator	Extreme compositions affecting flow rate (e.g. high lipid content)	Losses during sample preparation
Interferences leading to false positives	Cross contamination during sample preparation or incubation Extreme milk compositions (somatic cell count, protein, fat content have been reported to affect test results - to be assessed during validation)*	Preservatives or other additives with inhibitory action give false positive results High pH (>7), extreme composition, free fatty acids *Pseudonomiatoxins* can cause false-positive test results	Cross-reactivity vs. antibiotics from other families	Cross-reactivity vs. antibiotics from same or other families or matrix background signal when working close to LOD Matrix interferences
Validation level	Suppliers validate tests for selectivity, specificity, detection capability, robustness following official bodies guidelines. This data is available to end users	CRL guidelines for the validation of qualitative tests 2010/01/20[1]	CRL guidelines for the validation of qualitative screening tests 2010/01/20[2]	As detailed in the Commission Decision 2002/657/EC[2]

Source: IDF (2013a)

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* note: this is also true for confirmatory analysis

[1] GUIDELINES FOR THE VALIDATION OF SCREENING METHODS FOR RESIDUES OF VETERINARY MEDICINES, COMMUNITY REFERENCE LABORATORIES RESIDUES (CRLs), 20/1/2010 http://ec.europa.eu/food/food/chemicalsafety/residues/Guideline_Validation_Screening_en.pdf

[2] COMMISSION DECISION of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:en:pdf

With regard to microbial screening test sensitivity, studies carried out in sheep milk as well as goat's milk indicate that these methods are suitable for the detection of beta-lactam antibiotics but that they are not suitable for other antimicrobial groups such as tetracyclines, aminoglycosides, or quinolones, as the detection capability (CCβ) for these substances is higher than the MRLs established by legislation (Althaus et al., 2001a; Althaus et al., 2003a; Montero et al., 2005; Linage et al., 2007; Sierra et al., 2009a,b).

As for specificity of microbial screening tests, studies performed using sheep milk samples, found, in general, a large number of false-positive results, especially if milk samples are spiked with a preservative. Thus, Molina et al. (2003b), indicate a specificity of 96.3 and 97.7 % for the microbial screening tests BRT (AiM) and Delvotest SP (DSM Food Specialties), respectively, when antibiotic-free sheep milk samples were analysed without preservative. After the application of a heat treatment (85 °C for 10 min) to inactivate the natural inhibitors present in milk, an increase of test specificity, obtaining values of 99.0 and 98.7 %, respectively, was achieved, being very similar to those reported by Roca et al. (2007) for these same screening tests using milk from cows (99 and 98 % for the BRT and Delvotest SP, respectively). However, in sheep milk samples spiked with azidiol, Molina et al. (2003b) obtained lower specificity values (90.2 and 91 % for the BRT and Delvotest SP tests, respectively), even after the heat treatment (BRT: 94.8 % and Delvotest SP: 95.3 %), indicating that the growth of the microorganism-test was affected by the inhibitor effect of the preservative. In all case, azidiol interferences could be minimised by prolonging the incubation time (Zorraquino et al., 1997; Molina et al., 1999).

Most commercial microbial screening tests are based on milk diffusion through a culture medium, usually containing agar, nutrients and a standardized number of spores of a thermophile microorganism together with a dye type redox or acid-base indicator. However, the growth of this bacterium may be affected by other factors such as natural inhibitors present in milk (Carlsson and Björck, 1989), as higher somatic cell count (Cullor et al., 1992) or the presence of other substances such as detergents and disinfectants (Schiffmann et al., 1992).

Sheep milk contains a larger amount of natural inhibitors than cow milk (Althaus et al., 2001b; Park et al., 2007), which could explain the lower specificity values obtained for this species. Also, it presents a higher fat and protein contents than cow or goat's milk, complicating its diffusion through the culture medium content in the method, which has

also been linked to a larger number of questionable and positive results using the BRT and Delvotest tests (Althaus et al., 2003b). Also, higher SCC in sheep milk samples has been related to the occurrence of false-positive results in the same microbiological tests (Althaus et al., 2003b).

In goat's milk, just as in sheep milk, differences in the milk composition with respect to cow milk, also suggest that microbial inhibitor tests for the detection of antibiotics may not be suitable for this species (Hozová and Minarovičová, 2001). Thus for example, the elevated somatic cell count (SCC) in goat's milk with respect to cow milk even in the absence of intra-mammary infections (Paape et al., 2007; Mehdid et al., 2013), prompted Contreras et al. (1997) to examine whether this factor could interfere with the detection of antibiotic residues using the microbial inhibitor tests Charm BsDA, Delvotest P and Delvotest SP. These authors found that the three methods presented a specificity of 100 % (no positive results) in samples with high SCC as well as low SCC, which led the authors to suggest that they were appropriate for antibiotic screening in goat's milk. However, Zeng et al. (1996) by studying the performance of the Delvotest P test with goat's milk obtained 7 % false-positive results in Delvotest P in comparison with Charm BsDA acting as a reference test. Neither did these authors observe interferences due to SCC in the BsDA, Delvotest P and Delvotest SP microbiological methods.

The receptor-binding assays, known as rapid receptor tests, have been validated with cow milk samples showing higher specificity, lower false-positive rates and CCβs at or below MRLs for most antibiotics at which they are directed. Hence, Žvirdauskiene and Salomskiene (2007), indicated that the Betastar test (Neogen Corporation), a specific receptor-binding assay to detect beta-lactam residues in milk, was able to detect benzylpenicillin, oxacillin, ampicillin, at or below the MRLs set out in legislation. With this same rapid test and following a simplified trial procedure, Reybroeck et al. (2010) obtained a high sensitivity to different beta-lactam antibiotics in different types of milk (UHT, sterilized, reconstituted in powder, thawed) as well as a high specificity and a very small percentage of false-positive results. Also, Perme et al. (2010), obtained good sensitivity results with the Twinsensor[BT] test, a rapid assay for the simultaneous detection of beta-lactam and tetracycline residues in milk samples. However, data on related performance characteristics of rapid receptor tests in sheep and goat's milk are still rather limited.

In goat's milk, Contreras et al. (1997) indicated a specificity of 100 % (no false-positive results) when analysing individual milk samples from goats by the SNAP

Betalactam test (IDEXX Laboratories), a receptor-binding assay to detect beta-lactam residues in milk. Also, Zeng et al. (1998) obtained a specificity of 96.7 % for the same test using raw commingled goat's milk. In terms of sensitivity, the SNAP Betalactam test was able to detect amoxicillin, ampicillin, benzylpenicillin, cloxacillin, cephapirin and ceftiofur at or below safety limits (Tolerance/Salfe Levels) established by USFDA (FDA, 2005). However, hitherto no data on the performance of rapid receptor tests in sheep milk have been published. Neither have studies about the potential interference of the preservative azidiol on the response of this type of tests been carried out.

In spite of the lack of data concerning rapid receptor tests in these species, their implementation in the screening of sheep and goat's milk has increased in recent years, due to new legislative requirements and the need for fast results. However, as recommended by the E47 group "Antimicrobials and other veterinary residues" of the IDF, studies in sheep and goat's milk are to be continued to ensure good practices in this sector.

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Chapter 2

Objectives

Objectives

Antimicrobials can result in residues in foodstuffs of animal origin such as milk and therefore, it is necessary to establish an analytical strategy using appropriate methodologies for their detection and prevent them from reaching consumers. In Spain Real Decreto 752/2011 establishes the rules for the quality control of raw milk from sheep and goats, which includes the mandatory control of the presence of antibiotic residues in different steps of the production system.

Microbial screening tests are widely used in raw sheep and goat's milk quality control. As a result of the changes made in recent years to improve detection capability there are currently new versions available that have, however, not been evaluated with milk from small ruminants. On the other hand, specific receptor-binding tests are also routinely used for the rapid screening of beta-lactams in milk due to the new legislative requirements. Nevertheless, information about the suitability of rapid receptor tests using milk from small ruminants is very limited. Neither has the effect the preservative azidiol, authorized by Spanish regulation in milk sampling, can have on the response of receptor-binding tests been studied.

The evaluation of the currently available screening tests is essential in order to establish the most appropriate analytical strategy that allows detecting the greatest number of the substances most commonly used in the treatment of infectious diseases in dairy sheep and goats and ensure the safety of the milk and dairy products. Therefore, the following objectives of this thesis are:

Objective 1. To assess the microbial inhibitor tests for screening antibiotics in sheep and goat's milk.

Objective 2. To evaluate the receptor-binding assays for screening beta-lactam and tetracycline residues in sheep and goat's milk.

Objective 3. To determine the most appropriate analytical strategy in Spain for the detection of antimicrobial residues in sheep and goat's milk.

These objectives are pursued through various experiments presented in the Results section in the form of three chapters corresponding to each of the three objectives established.

Chapter 3

Assessment of microbial inhibitor tests for screening antibiotics in sheep and goat's milk

Performance of current microbial tests for screening antibiotics in sheep and goat's milk

Beltrán M.C.[a], Berruga M.I.[b], Molina A.[b], Althaus R.L.[c], Molina M.P.[a]

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

[b]Departamento de Ciencia y Tecnología Agroforestal, ETSIA-IDR, Universidad de Castilla-La Mancha, 02071 Albacete, Spain.

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

Abstract

The performance of different microbial tests currently employed for screening antibiotic residues in milk was investigated using sheep and goat milk. Detection capability (CCβ) of the BRT MRL, Delvotest MCS SP-NT, Delvotest MCS Accelerator, and the Eclipse 100 tests was calculated according to European Commission Decision 657/2002. All the tests were able to detect most β-lactam antibiotics assessed in sheep as well as goat milk. For non-β-lactam drugs, only 20 % of substances were detected at or below their respective maximum residue limits (MRL). Microbial screening tests showed an elevated percentage of non-compliant outcomes (4.8-10 %) when antimicrobial-free sheep milk samples were analysed. The positive results were related to elevated somatic cell counts (SCC) in milk. However, test responses were unaffected by goat milk properties when individual antimicrobial-free milk samples were checked along the entire lactation, and false-positive outcomes recorded were below 5 % in all cases. In conclusion, microbial screening tests are an efficient tool for the detection of β-lactams in raw milk from sheep and goats. Nevertheless, they are inefficient to detect most non-β-lactam drugs employed by veterinarians for therapeutic and prophylactic treatments of infectious diseases in ovine and caprine livestock. Therefore, the periodical use of more sensitive microbial methods for these substances or the application of specific methods on a different analytical basis would be convenient. Thus, the detection range in screening could be widened and the safety of milk and dairy products from small ruminants would be guaranteed.

1. Introduction

Microbial inhibitor tests are routinely applied in the control of the presence of antibiotic residues in raw milk as they are relatively inexpensive, user-friendly, and able to detect a great variety of antimicrobials in a large number of milk samples.

Most current microbial screening tests were initially developed to detect beta-lactams in cow milk and are based on the inhibition of *Geobacillus stearotermophilus* var. *calidolactis* being highly sensitive to these substances. Efforts to improve test sensitivity, in particular towards non-beta-lactam drugs, has been made by different authors who proposed some modifications such as the addition of chelating agents or antifolates, such as trimethoprim, into the culture medium, to enhance the detection of tetracyclines and sulphonamides in milk, respectively (Adriany et al.,1995; Langeveld et al., 2005). In recent years, manufacturers have improved some performance characteristics of microbial methods, especially the time required for the analyses, and sensitivity to different substances, and new versions of these tests are now available (ISO/IDF, 2010).

Different studies on the sensitivity of microbial screening tests have been carried out in the last two decades by different researchers using sheep and goat's milk (Althaus et al., 2003a; Molina et la., 2003; Sierra et al., 2009a,b), demonstrating that these tests are able to detect beta-lactams at or below the maximum residue limits (MRLs) established by European legislation (EC, 2010), however they are not suitable for other drugs.

Although beta-lactams are widely used to treat mastitis and other infections in dairy sheep and goats, other antimicrobials like tetracyclines, quinolones or aminoglycosides, are often used for therapeutic and prophylactic treatments in these species. Therefore, knowledge of the detection capabilities (CCβs) of currently available microbial tests for these substances is relevant to assure the safety of milk of these species.

Moreover, sheep and goat milk is characterized by a higher fat and protein content than cow milk (Park et al., 2007) as well as by an elevated content of natural inhibitors such as immunoglobulins, lactoferrin, or lysozyme (Crosson et al., 2010) and higher somatic cell count (SCC) even in absence of intra-mammary infections (Paape et al., 2007) that might interfere in the response of microbial inhibitor tests. In fact, some authors have also studied the specificity of several microbial tests obtaining higher false non-compliant rates when using individual sheep's milk samples (Althaus

et al., 2003b). In goat milk, information about the effect of the milk characteristics on the microbial test response is more limited (Contreras et al., 1997).

Antibiotic residues have negative repercussions on the technological properties of milk as they can totally or partially inhibit fermentation procedures required when making cheese and yoghurt (Packham et al., 2001). Sheep and goat milk is basically destined for the elaboration of fermented products, and antibiotics in milk can thus affect the production process; also, as residues of variable amounts may remain in the final products, their consumer safety might be compromised (Oliver et al., 2011); therefore it seems necessary to establish control measures to rule out the presence of these substances above legally established limits, employing suitable screening tests for this purpose.

The aim of this study was to assess the performance of new versions of some microbial screening tests to detect antimicrobial residues in sheep and goat milk according to European Commission Decision 657/2002 (EC, 2002).

2. Material and methods

2.1. Microbial inhibitor tests

The screening tests used were the BRT MRL (Analytik in Milch Produktions-und 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). All tests were conducted according to the each manufacturer's instructions. Negative (antibiotic-free milk) and positive (antibiotic-free milk spiked with 4µg/Kg of benzylpenicillin) control samples were also included in all the experimental plates. Interpretation of the test results were carried out independently by three trained technicians assessing visually the colour change after incubation, and classifying milk samples as positive (blue) or negative (yellow). Samples showing a doubtful colour change were classified as questionable. For the Delvotest MCS Accelerator (DA) test, the results were classified instrumentally using the Delvotest Accelerator device (DSM Food Specialties).

2.2. Milk samples

Antibiotic-free milk samples were obtained from the experimental flocks of Manchega ewes 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

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before nor along the experimental period. Neither was medicated feed used in their diet.

All milk samples were analysed for gross composition (MilkoScan 6000, Foss. Hiller∅d, Denmark), somatic cell count (Fossomatic 5000, Foss), total bacterial count (Bactoscan FC, Foss), and pH value (pHmeter, Crison, Barcelona, Spain).

2.3. Antimicrobials and spiked milk samples

The list with the total antimicrobial substances assayed is shown in Table 1. 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, 2003), and tested simultaneously applying the microbial screening tests immediately after spiking.

2.4. Detection capability (CCβ)

Detection capability (CCβ) of the four microbial screening tests was investigated following the "Guidelines for the validation of screening methods for residues of veterinary medicines" proposed by Community Reference Laboratories for residues (CRLs, 2010), which supplements Commission Decision nº 657/2002 (EC, 2002) and defines the CCβ as the lowest antibiotic concentration assessed which produces at least 95 % positive results (false compliant results ≤ 5 %). To calculate the CCβ of microbial screening tests, antimicrobial-free milk samples from sheep and goats were collected in the mid-lactation period to be used as "negative milk" as, according to the recommendations of the International Dairy Federation (ISO/IDF, 2003), such samples show good hygienic quality and a characteristic gross composition.

Antimicrobial-free milk samples were spiked individually with different substances at 0.5xMRL, 0.75xMRL, and 1xMRL equivalent drug concentration, and analysed 20, 40 or 60 times, respectively, by the different microbial tests.

2.5. Interferences related to milk matrix constituents

To investigate the effect of the milk constituents on the microbial test response, individual milk samples free of antimicrobials collected periodically thorough the milking period were used. Sheep milk samples (n= 250) were obtained on a two-week basis at the morning milking from the first week after weaning until the end of lactation (sampling: days 35 to 170 post-partum). Goat milk samples (n= 350) were also collected every two weeks from the second week post-partum during a period of seven months (sampling: days 15 to 200 post-partum).

Table 1. Antimicrobials used to evaluate microbial screening tests in sheep and goat milk

Antimicrobials	Distributor	Commercial reference	Solvent
Beta-lactams			
Amoxicillin	Sigma-Aldrich[a]	A8523	H_2O
Ampicillin	Sigma-Aldrich[a]	A9518	H_2O
Benzylpenicillin	Sigma-Aldrich[a]	PENNA	H_2O
Cloxacillin	Sigma-Aldrich[a]	C9393	H_2O
Dicloxacillin	Sigma-Aldrich[a]	D9016	MeOH / H_2O
Nafcilin	Sigma-Aldrich[a]	N3269	MeOH / H_2O
Oxacillin	Sigma-Aldrich[a]	46589	MeOH / H_2O
Cefacetrile	Fatro[b]	*	H_2O
Cefalonium	Sigma-Aldrich[a]	32904	NaOH 0.1N /H_2O
Cefapirin	Sigma-Aldrich[a]	43989	H_2O
Desacetylcefapirin	ACS Dobfar[c]	*	H_2O
Cefazolin	Sigma-Aldrich[a]	C5020	H_2O
Cefoperazone	Sigma-Aldrich[a]	32426	NaOH 1N / H_2O
Cefquinome	Sigma-Aldrich[a]	32472	H_2O
Ceftiofur	Sigma-Aldrich[a]	34001	NaOH 0.1N / H_2O
Desfuroylceftiofur	TRC[d]	D289980	MeOH / H_2O
Cephalexin	Sigma-Aldrich[a]	C4895	H_2O
Aminoglycosides			
Gentamicin	Sigma-Aldrich[a]	G3632	H_2O
Neomycin	Sigma-Aldrich[a]	N1876	H_2O
Streptomycin	Sigma-Aldrich[a]	S6501	H_2O
Macrolides			
Erythromycin	Sigma-Aldrich[a]	E6376	EtOH / H_2O
Lincomycin	Sigma-Aldrich[a]	31727	H_2O
Tylosin	Sigma-Aldrich[a]	T6271	H_2O
Quinolones			
Enrofloxacin	Sigma-Aldrich[a]	33699	AcOH 5% / H_2O
Ciprofloxacin	Sigma-Aldrich[a]	17850	HCl 0.1N
Marbofloxacin	Sigma-Aldrich[a]	34039	H_2O
Sulphonamides			
Sulfadiazine	Sigma-Aldrich[a]	S6387	H_2O
Sulfadimethoxine	Sigma-Aldrich[a]	S7385	H_2O
Sulfametazine	Sigma-Aldrich[a]	S5637	H_2O
Tetracyclines			
Chlortetracycline	Sigma-Aldrich[a]	C4881	NaOH 0.1N / H_2O
4-epichlortetracycline	Acros[e]	268235000	MeOH / H_2O
Oxytetracycline	Sigma-Aldrich[a]	O4636	HCl 0.1N / H_2O
4-epioxytetracycline	Acros[e]	25771	MeOH / H_2O
Tetracycline	Sigma-Aldrich[a]	T3258	HCl 0.1N / H_2O
4-epitetracycline	Acros[e]	233125000	MeOH / H_2O
Others			
Colistin	Sigma-Aldrich[a]	C4461	H_2O
Trimethoprim	Sigma-Aldrich[a]	92131	EtOH

[a]Sigma-Aldrich Química, S.A. (Madrid, Spain)
[b]Fatro, S.p.A. (Bologna, Italy)
[c]ACS Dobfar, S.p.A. (Milan, Italy)
[d]Toronto Research Chemicals, Inc. (Toronto, Canada)
[e]Acros Organics (Geel, Belgium)
*Commercial reference not available

All samples were analysed simultaneously, in three replicates, by the four tests, and non-compliant outcomes were recorded as interferences. Samples showing positive and questionable results in at least two replicate analyses were finally recorded as non-compliant results.

2.6. Statistical analysis

To investigate the effect of the milk matrix constituents on the microbial test response, a logistic regression model was applied. Statistical analysis was carried out employing the stepwise option of the logistic procedure of the SAS software (version 9.2, 2001; SAS Institute, Inc., Cary, NC), using the following logistic model:

$$L_{ij} = \text{logit } [P_i] = \beta_0 + \beta_1[SL] + \beta_2[pH] + \beta_3[F] + \beta_4[P] + \beta_5[L] + \beta_6[TS] + \beta_7[\log SCC] + \beta_8[\log BC] + \varepsilon_{ij} \text{ (Eq. 1)}$$

where: L_{ij} is the logistic model; $[P_i]$ is the probability for the response category (positive/negative); β_0 is the intercept; β_i are the estimate parameters for the model; [SL] is the lactation stage effect (day); [pH] is the pH effect; [F] is the fat content effect; [P] is the protein content effect; [L] is the lactose content effect; [TS] is the total solids content effect; [logSCC] is the somatic cell count effect; [logBC] is the bacterial count effect; ε_{ij} is the residual error.

3. Results and discussion

3.1. Detection capability (CCβ)

The CCβ of the BRT MRL, Delvotest MCS SP-NT, Delvotest MCS DA, and Eclipse 100 tests in sheep milk were shown in Table 2. On average, these tests allow the detection of 83.8 % beta-lactam substances at or below the required safety level (70.6 %: BRT MRL, and 88.2 %: Delvotest MCS SP-NT, Delvotest MCS DA, and Eclipse 100). Only cefquinome and cefoperazone could not be detected by any test at MRL equivalent antibiotic concentration. Moreover, the BRT MRL test could neither detect ceftiofur, its metabolite desfuroylceftiofur, and cephalexin at their respective MRL. In goat milk, the CCβ results for beta-lactam substances (Table 3) were similar to those previously reported for sheep milk (76.4 %: BRT MRL, and 82.3 %: Delvotest MCS SP-NT; Delvotest MCS DA, and Eclipse 100) the BRT MRL test being the least sensitive for the detection of these substances.

The sensitivity of microbial tests to detect beta-lactams has improved in the last years. Thus, for instance, the detection levels of these tests for widely used antibiotics such as benzylpenicillin or ampicillin was above MRLs (Heeschen and Blüthgen, 1991)

Table 2. Detection capability (CCβ) of microbial screening tests for the detection of antimicrobials in sheep milk

Antimicrobial	MRL[a] (µg/Kg)	CCβ[b] (µg/Kg)			
		BRT MRL	Delvotest SP-NT	Delvotest DA	Eclipse 100
Beta-lactams					
Amoxicillin	4	4	3	3	3
Ampicillin	4	3	3	3	4
Benzylpenicillin	4	3	3	3	4
Cefacetrile	125	≤ 63	≤ 63	≤ 63	≤ 63
Cefalonium	20	20	≤ 10	20	20
Cefapirin	60	≤ 30	≤ 30	≤ 30	≤ 30
Deacetylcefapirin	*	≤ 30	≤ 30	≤ 30	≤ 30
Cefazolin	50	≤ 25	≤ 25	≤ 25	≤ 25
Cefoperazone	50	> 50	> 50	> 50	> 50
Cefquinome	20	> 20	> 20	> 20	> 20
Ceftiofur	100	> 100	100	100	100
Desfuroylceftiofur	*	> 100	75	100	100
Cephalexin	100	> 100	≤ 50	≤ 50	≤ 50
Cloxacillin	30	23	≤ 15	≤ 15	23
Dicloxacillin	30	≤ 15	≤ 15	≤ 15	23
Nafcillin	30	≤ 15	≤ 15	≤ 15	≤ 15
Oxacillin	30	≤ 15	≤ 15	≤ 15	≤ 15
Aminoglycosides					
Gentamicin	100	100	>100	>100	>100
Neomycin	1,500	≤ 750	≤ 750	≤ 750	> 1,500
Streptomycin	200	> 200	> 200	> 200	> 200
Macrolides					
Erythromycin	40	40	> 40	> 40	> 40
Lincomycin	150	> 150	> 150	> 150	> 150
Tylosin	50	≤ 25	≤ 25	≤ 25	≤ 25
Quinolones					
Enrofloxacin	100	> 100	> 100	> 100	> 100
Ciprofloxacin	*	> 100	> 100	> 100	> 100
Marbofloxacin	75	> 75	> 75	> 75	> 75
Sulphonamides					
Sulfadiazine	100	>100	75	75	75
Sulfadimethoxine	100	≤ 50	≤ 50	75	100
Sulfametazine	100	> 100	>100	>100	> 100
Tetracyclines					
Chlortetracycline	100	>100	>100	>100	>100
4-epichlortetracycline	*	>100	>100	>100	>100
Oxytetracycline	100	>100	>100	>100	>100
4-epichlortetracycline	*	>100	>100	>100	>100
Tetracycline	100	>100	>100	>100	>100
4-epitetracycline	*	>100	>100	>100	>100
Others					
Colistin	50	> 50	> 50	> 50	> 50
Trimethoprim	50	> 50	> 50	> 50	> 50

[a]MRL: Maximum Residue Limit (µg/Kg) established by EC Regulation Nº 37/2010 (EC, 2010); [b]CCβ: Detection Capability (lower antimicrobial concentration which produces at least 95 % positive results); *: marker residue. MRL not established

Table 3. Detection capability (CCβ) of microbial screening tests for the detection of antimicrobials in goat milk

Antimicrobial	MRL[a] (µg/Kg)	CCβ[b] (µg/Kg)			
		BRT MRL	Delvotest SP-NT	Delvotest DA	Eclipse 100
Beta-lactams					
Amoxicillin	4	3	4	4	4
Ampicillin	4	2	2	3	4
Benzylpenicillin	4	2	2	2	2
Cefacetrile	125	≤ 63	≤ 63	≤ 63	≤ 63
Cefalonium	20	15	15	15	15
Cefapirin	60	≤ 30	≤ 30	≤ 30	≤ 30
Deacetylcefapirin	*	≤ 30	≤ 30	≤ 30	≤ 30
Cefazolin	50	≤ 25	≤ 25	≤ 25	≤ 25
Cefoperazone	50	> 50	> 50	> 50	> 50
Cefquinome	20	> 20	> 20	> 20	> 20
Ceftiofur	100	> 100	> 100	> 100	> 100
Desfuroylceftiofur	*	100	100	100	100
Cephalexin	100	> 100	75	≤ 50	≤ 50
Cloxacillin	30	23	23	23	23
Dicloxacillin	30	≤ 15	≤ 15	≤ 15	≤ 15
Nafcillin	30	≤ 15	≤ 15	≤ 15	≤ 15
Oxacillin	30	≤ 15	≤ 15	≤ 15	≤ 15
Aminoglycosides					
Gentamicin	100	100	>100	>100	>100
Neomycin	1,500	≤ 750	≤ 750	≤ 750	> 1,500
Streptomycin	200	> 200	> 200	> 200	> 200
Macrolides					
Erythromycin	40	40	> 40	> 40	> 40
Lincomycin	150	> 150	> 150	> 150	> 150
Tylosin	50	50	≤ 25	50	50
Quinolones					
Enrofloxacin	100	> 100	> 100	> 100	> 100
Ciprofloxacin	*	> 100	> 100	> 100	> 100
Marbofloxacin	75	> 75	> 75	> 75	> 75
Sulphonamides					
Sulfadiazine	100	>100	≤ 50	75	≤ 50
Sulfadimethoxine	100	≤ 50	≤ 50	≤ 50	100
Sulfametazine	100	> 100	> 100	>100	> 100
Tetracyclines					
Chlortetracycline	100	>100	>100	>100	>100
4-epichlortetracycline	*	>100	>100	>100	>100
Oxytetracycline	100	>100	>100	>100	>100
4-epichlortetracycline	*	>100	>100	>100	>100
Tetracycline	100	>100	>100	>100	>100
4-epitetracycline	*	>100	>100	>100	>100
Others					
Colistin	50	> 50	> 50	> 50	> 50
Trimethoprim	50	> 50	> 50	> 50	> 50

[a]MRL: Maximum Residue Limit (µg/Kg) established by EC Regulation Nº 37/2010 (EC, 2010); [b]CCβ: Detection Capability (lower antimicrobial concentration which produces at least 95 % positive results); *: marker residue. MRL not established

while they are currently detected at or below legally stipulated levels (Stead et al., 2008). However, their sensitivity for some cephalosporins such as cefquinome, cephalexin or cefoperazone remains still inadequate (Sierra et al., 2009a). It should be noted that within the beta-lactam antibiotics family, penicillins are most widely used by veterinarians to treat mastitis in dairy small ruminants (Ferrini et al., 2010), cephalosphorins being less used in these species. Therefore the sensitivity results obtained in this study confirm the suitability of microbial inhibitor tests for screening residues of penicillins in sheep and goat milk.

For non-beta-lactam drugs, the CCβ was above MRL for all antimicrobial substances except for neomycin, tylosin and sulfadimetoxine that were detected in sheep milk at or below regulatory limits (Table 2). In contrast to the other tests, the BRT MRL test was also able to detect gentamicin at erythromycin at their MRLs. In goat milk, the CCβ for antimicrobial families other than β-lactams was also above their MRLs in most cases (Table 3). Thus, microbial inhibitor tests only allowed, on average, the detection of 20 % of non-beta-lactam drugs at or below the safety level in sheep milk as well in goat milk (25 %: BRT MRL, 20 %: Delvotest MCS SP-NT, and Delvotest MCS DA, and 15 %: Eclipse 100). Although manufacturers have improved the sensitivity of microbial inhibitor tests for certain drugs, they still remain inefficient for the detection of antibiotics such as tetracyclines, quinolones, and aminoglycosides (Sierra et al., 2009b), usually employed in the therapy and veterinary prophylaxis in ovines and caprines, representing a potential food safety risk.

3.2. Interferences related to the milk matrix effect

To study the effect of milk composition on the microbial test response, individual milk samples free of antimicrobials were collected throughout the lactation period. Milk samples presented a wide range of variation in milk quality parameters (Table 4).

Microbial screening tests showed an elevated percentage of non-compliant results (up to 10 %) when antimicrobial-free sheep milk samples were assayed (Table 5). For this species, logistic regression analysis showed that an increase in SCC was associated with an elevation in the predicted likelihood of positive outcomes for all the microbial screening tests (Figure 1), in which the BRT MRL test response was the least affected by this parameter. These results were in agreement with those obtained by others (Cullor et al., 1992) who also observed a significant effect of SCC on the frequency of positive results for different screening methods using individual milk samples from cows.

Table 4. Quality parameters of individual milk samples free of antimicrobials used to evaluate the effect of the matrix constituents on the microbial test response

Parameter	Individual sheep milk (n=250)				Individual goat milk (n=350)			
	Mean	SD[a]	Min[b]	Max[c]	Mean	SD	Min	Max
pH	6.66	0.08	6.54	6.92	6.79	0.09	6.25	7.02
Fat (%)	6.38	2.05	2.42	12.68	5.57	1.15	3.23	9.59
Protein (%)	5.83	0.73	4.55	7.82	3.74	0.49	2.44	5.36
Lactose (%)	5.03	0.35	3.87	5.67	4.65	0.31	2.90	5.38
Total solids (%)	18.06	2.71	12.51	26.53	14.66	1.53	11.05	19.48
Log SCC[d]	5.01	0.48	4.00	7.27	5.77	0.42	4.88	7.19
Log BC[e]	4.83	0.72	3.78	6.96	4.55	0.40	4.00	6.36

[a]SD: standard deviation; [b]Min: minimum; [c]Max: maximum; [d]Log SCC: logarithm of somatic cell count; [e]Log BC: logarithm of bacterial count

It is important to mention that in control quality programmes raw bulk milk samples are usually analysed, not individual milk, presenting a minor range of variation in all quality parameters and very low percentages of non-compliant results (Comunian et al., 2010). However, the test responses were unaffected by goat milk properties, including SCC, when antimicrobial-free milk samples were analysed; and the false-positive outcomes recorded were below 5 % in all cases (Table 5). These results could be related with the particularity of goats in which high SCCs might be due to non-infectious factors such as stress or oestrus, among others, which do not lead to modifications in plasma-component concentration of known antimicrobial activity, characteristic of mastitis processes (Andrew et al., 1997).
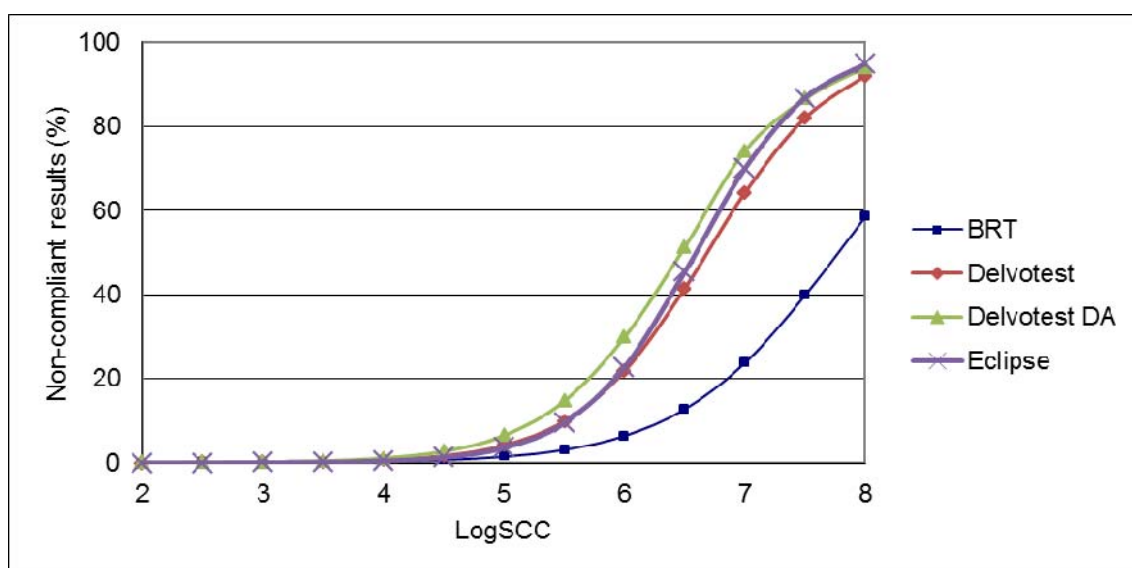

Figure 1. Effect of the somatic cell count (SCC) on the false non-compliant outcomes of microbial screening tests using sheep milk

Table 5. Microbial inhibitor test results in individual sheep and goat milk samples free of antimicrobials

Microbial tests	Sheep milk (n=250)				Goat milk (n=350)			
	P[a]	Q[b]	N[c]	C[d] (%)	P	Q	N	C (%)
BRT MRL	7	5	238	95.2	1	4	345	98.6
Delvotest MCS SP-NT	11	9	230	92	6	5	339	96.9
Delvotest MCS DA[*]	25	-	225	90	15	-	335	95.7
Eclipse 100	10	14	226	90.4	1	1	348	99.4

[a]P: positive; [b]Q: questionable; [c]N: negative; [d]C (%): percentage of compliant outcomes; *: only two categories of test results in the Delvotest MCS DA

4. Conclusions

Microbial inhibitor tests using *Geobacillus stearothermophilus* var. *calidolactis* as a microorganism are efficient to detect beta-lactams in raw milk from small ruminants avoiding that such substances reach consumers. However, in spite of the improvements made in these tests along the last decade, they continue to be inefficient for other drugs, such as tetracyclines, quinolones or macrolides, usually employed for therapeutic and prophylactic treatments in dairy livestock. Therefore, the periodical use of more sensitive microbial methods towards these substances or the application of specific methods on different analytical bases would be convenient, which would widen the detection range in screening and guarantee the quality of milk and dairy products from small ruminants.

5. Acknowledgments

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Novel microbiological system for antibiotic detection in ovine milk

Nagel O.G.[a], Beltrán M.C.[b], Molina M.P.[b], Althaus R.L.[a]

[a]Cátedra de Biofísica. Facultad de Ciencias Veterinarias. Universidad Nacional del Litoral. R.P.L. Kreder 2805. 3080 Esperanza (Argentina)

[b]Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera, s/n. 46022 Valencia (Spain).

Abstract

This article presents a microbiological system composed of a "BT" bioassay (Beta-lactams and Tetracyclines) and a "QS" bioassay (Quinolones and Sulfonamides). The "BT" bioassay contains spores of *Geobacillus stearothermophilus*, bromocresol purple and cloramphenicol in a culture medium (incubation time: 2.45 h), while the "QS" bioassay uses spores of *Bacillus subtilis*, trifenyltetrazolium - toluidine blue and trimethoprim in a suitable culture medium (incubation time: 5.5 h). The detection capability (CCβ) of 27 antimicrobial agents in ovine milk was determined by logistic regression models. Thus, the "BT" bioassay detects amoxicillin, ampicillin, benzylpenicillin, cloxacillin, oxacillin, cephalexin, cefoperazone, ceftiofur, chlortetracycline, oxytetracycline, tetracycline, neomycin, gentamicin and tylosin, while "QS" bioassay detects: ciprofloxacin, enrofloxacin, marbofloxacin, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfathiazole, erythromycin, lincomycin and spiramycin at levels close to their respective maximum residue limits (MRLs). The simultaneous use of both bioassays detects a large number of antibiotics in milk given each method's adequate complementary sensitivity.

Keywords: ovine milk; system; bioassay; detection

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1. Introduction

In recent years, increased use of antibiotics to treat mastitis and other diseases of small ruminants was observed due to the intensification of milk production (Buswell and Barber, 1989).

The presence of antibiotic residuals in milk poses a potential risk for the consumers as they may cause allergic type reactions, and may interfere with intestinal flora and the development of resistance to antibiotics (Demoly and Romano, 2005; Dewdney et al., 1991; Currie et al., 1998; Wilke et al., 2005). Furthermore, antibiotic residues in milk can lead to important losses in fermented products, such as cheese-making (Mourot and Loussouorn, 1981; Brady and Katz, 1988; Packham et al., 2001; Berruga et al., 2007).

Therefore, monitoring antibiotic residues is very important in controlling food safety. For these reasons, several control authorities such as the European Union (Council Directive, 2009) and Codex (Codex Alimentarius, 2009) determine the Maximum residue limit (MRL) for the presence of specified veterinary residues in milk.

To this end, several commercially available tests have been developed for the swiftly and precisely detect of the presence of antibiotic residuals in milk (Toldra and Reig, 2006). Many of the screening tests are based on the inhibition of *Geobacillus stearothermophilus* var. *calidolactis* caused by the presence of drug residues. However, this bacteria does not have sensitivity to detect many of the antibiotics used to treat livestock such as quinolones (Montero et al., 2005), spiramycin, lincomycin (Linage et al., 2007), erythromycin and streptomycin (Molina et al., 2003; Althaus et al., 2002, 2003).

In addition, rapid methods are specific to small groups of antibiotics, but cannot increase the number of molecules to be controlled (Althaus et al., 2001; Roca et al., 2009).

Given the absence of a single ideal screening method that is sensitive to a large number of antimicrobial agents in ovine milk, the objective of this study was to evaluate the application of a microbiological system that uses two bacteria test (*Geobacillus stearothermophilus* and *Bacillus subtilis*) to detect a larger number of antibiotics in milk and to ensure consumer food safety.

2. Materials and methods

2.1. Preparation of microplates

The "BT" bioassay (*G. stearothermophilus*): Plate Count Agar (Difco, Ref. 247940) culture medium (6.25 g/l casein peptone, 2.25 g/l yeast extract and 15 g/l agar) fortified with glucose (10 g/l; Sigma, Ref 158968) was used. The culture medium was sterilized to 121 ºC for 15 min. Then, it was cooled to 50±1 ºC and the pH was adjusted to a value of 7.0±0.1. Once prepared, the spores suspension of *G. stearothermophilus* var. *calidolactis* C-953 (10^7 spores/ml, Merck, Ref. 1.11499), bromocresol purple indicator (0.05 mg/l, Mallinckrodt, Ref. 2090) and chloramphenicol (400 μg/ml, Sigma Aldrich, Ref. C0378) were added in accordance with Nagel et al. (2009).

The "QS" bioassay (*B. subtilis*): Müeller Hinton (38 g/l, Biokar Diagnostics, Ref. BK048HA) culture medium fortified with glucose (10 g/l; Sigma Aldrich, Ref. G7528), trimethoprim (400 mg/l; Sigma Aldrich, Ref. T7883), 2,3,5-tripheyltetrazolium chloride (150 mg/l; Sigma Aldrich, Ref. T8877) and toluidine blue (15 mg/l; Sigma Aldrich, Ref. 198161) was employed. Once prepared, the culture medium was inoculated with the spore's suspension of *B. subtilis* BGA (Merck, Ref. 1.10649) under sterile conditions in accordance with Nagel (2009).

Then 100 μl of the culture medium were added to each individual well of microtiter plate using an electronic pipette (Eppendorf Research Pro). Next, these microplates were sealed with aluminized film and conserved at 4 ºC until use.

2.2. Animals and ewe milk samples

The ewes were fed with natural pastures of *Melilotus albus*, *Trifolium repens* and *Lolium multiflorum*, during the lactation period. Individual samples were collected from 40 Pampinta (Milchschaff x Corriedale) ewes from the experimental farm at the Escuela de Agricultura Ganadería y Granja of the Universidad Nacional del Litoral in Argentina (south latitude: 31° 28', west longitude: 60° 55'). Animals did not receive any antimicrobial substances, and the samples were collected from ewes in the period between 30 and 90 days postpartum, from the recorder jar during morning milking and placed in 100 ml sterile plastic containers. Milk samples were kept at 4 ºC throughout the experiment.

2.3. Antimicrobial solutions and spiked samples

Drugs for the preparation of antimicrobial solutions were stored and handled according to the manufacturers' instructions before use. All the dilutions were prepared in 10 ml volumetric flasks at the time when analyses were carried out to avoid possible inconvenience due to instability. Antimicrobial solutions were prepared from the respective stock solution in a single step using antimicrobial-free milk (IDF, 2002), as determined by the "BT" and "QS" bioassays.

The dose-response curves of the antimicrobial agents were established in line with the Codex Alimentarius guidelines (Codex Alimentarius, 2010). To this end, 8 concentrations were prepared with different levels of each drug (Table 1). For each concentration, 24 replicates were prepared using antibiotic-free ovine milk samples obtained from individual animals. Then, 50 μl milk samples were added to the individual wells of the "BT" and "QS" Bioassays. Plates were sealed with adhesive bands and incubated at 64$\pm$1 °C for 2.5 h ("BT" Bioassay) and 40$\pm$1 °C for 5.5 h ("QS" Bioassay) according to the colour change of the negative samples. Visual interpretation was carried out by 3 qualified individuals and evaluated as "negative" (BT" bioassay: yellow and "QS" bioassay: rose) or "positive" (BT" bioassay: purple and "QS" bioassay: blue).

For the statistical calculations, those visual results that presented at least 2 similar interpretations were considered.

2.4. Detection capability (CCβ) and statistical analysis

To determine the detection capability (CCβ), 8 beta-lactams (amoxicillin, ampicillin, benzylpenicillin, cloxacillin, oxacillin, cephalexin, cefoperazone, ceftiofur), 3 aminoglycosides (gentamicin, neomycin, streptomycin), 4 macrolides (erythromycin, lincomycin, tylosin, spiramycin), 3 quinolones (ciprofloxacin, enrofloxacin, marbofloxacin), 6 sulphonamides (sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfathiazole) and 3 tetracyclines (chlortetracycline, oxytetracycline, tetracycline) were analyzed according to Codex Alimentarius guidelines (Codex Alimentarius, 2010).

The results were obtained using the SAS Logistic procedure (SAS, 2001). The logistic regression model was also used to calculate the detection limits, as follows:

$$L_{ij} = \text{logit} [P_i] = \beta_0 + \beta_1 [A]_i + \varepsilon_{ij} \qquad (1)$$

where:

L_{ij} = lineal logistic model; $[P_i]$ = logit $[Pp/(1-Pp)]$: the probability of a "positive" response / probability of a "negative" response); β_0, β_1 = the coefficients estimated for the logistic regression models; $[A]_i$ = antimicrobial concentration. ε_{ij} = residual error.

The concordance coefficient (SAS, 2001) was applied as a rank correlation between the observed responses and the predicted probabilities.

Table 1. Antimicrobial agent concentrations using for microbiological system

Antibiotics	"BT" bioassay	"QS" bioassay
Beta-lactams		
Amoxicillin	0, 1, 2, 3, 4, 5, 6, 8	0, 1, 2, 4, 6, 8, 10, 12
Ampicillin	0, 1, 2, 3, 4, 5, 6, 8	0, 5, 10, 20, 30, 40, 50, 60
Benzylpenicillin	0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0	0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0
Cloxacillin	0, 10, 15, 20, 25, 30, 40, 60	0, 50, 100, 150, 200, 250,300, 400
Oxacillin	0, 2, 5, 10, 15, 20, 25, 30	0, 25, 50, 100, 150, 200, 250, 300
Cephalexin	0, 25, 50, 75, 100, 150, 200, 300	0, 25, 50, 100, 125, 150, 200, 300
Cefoperazone	0, 50, 75, 100, 150, 200, 300, 400	0, 50, 100, 125, 150, 200, 300, 400
Ceftiofur	0, 25, 50, 75, 100, 150, 200, 300	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8*
Aminoglycosides		
Gentamicin	0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8*	0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0*
Neomycin	0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0*	0, 2, 3, 4, 5, 6, 7, 8*
Streptomycin	0, 1, 2, 3, 4, 5, 6, 7*	0, 1, 2, 3, 4, 5, 6, 7*
Macrolides		
Erythromycin	0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4*	0, 10, 20, 30, 40, 50, 60, 70
Lincomycin	0, 0.1, 0.15, 0.20, 0.25, 0.30, 0.40, 0.50*	0, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, 0.50
Tylosin	0, 25, 50, 75, 100, 125, 150, 200	0, 60, 80, 100 ,120, 140, 160, 180
Spiramycin	0, 1, 2, 3, 4, 5, 6, 7*	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7*
Quinolones		
Ciprofloxacin	0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0*	0, 50, 100, 150, 200, 250, 300, 400
Enrofloxacin	0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0*	0, 50, 100, 150, 200, 250, 300, 400
Marbofloxacin	0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0*	0, 50, 100, 150, 200, 250, 300, 400
Sulphonamides		
Sulfadiazine	0, 10, 20, 30, 40, 50, 60, 80*	0, 50, 100, 200, 300, 400, 500, 600
Sulfadimethoxine	0, 0.5, 0.8, 1.0, 1.3, 1.5, 2.0, 3.0*	0, 50, 100, 200, 300, 400, 500, 600
Sulfamerazine	0, 10, 15, 20, 25, 30, 35, 40*	0, 50, 100, 200, 300, 400, 500, 600
Sulfamethazine	0, 10, 20, 30, 40, 50, 60, 80*	0, 50, 100, 200, 300, 400, 500, 600
Sulfamethoxazole	0, 5, 10, 15, 20, 25, 30, 35*	0, 50, 100, 200, 300, 400, 500, 600
Sulfathiazole	0, 5, 10, 15, 20, 25, 30, 35*	0, 50, 100, 200, 300, 400, 500, 600
Tetracyclines		
Clortetracycline	0, 50, 100, 150, 200, 300, 400, 500	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7*
Oxytetracycline	0, 25, 50, 75, 100, 150, 200, 300	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7*
Tetracycline	0, 25, 50, 75, 100, 150, 200, 300	0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2*

Units: μg/l or *mg/l

The CCβ were estimated as concentrations at which 95 % of the positive results (Codex Alimentarius, 2010).

3. Results and discussion

Table 2 shows the results obtained by applying the logistic regression model to the visual interpretations of the "BT" and "QS" bioassays for the 27 antimicrobials analyzed in sheep milk.

The "β_1" parameters indicate the slopes of the dose-response curves. Therefore, high values of this coefficient show a good sensitivity of the bacteria test to detect a particular antibiotic in milk.

The "BT" bioassay presents high "β_1" coefficients values to beta-lactam antibiotics, tetracyclines, tylosin and neomycin, while the "QS" bioassay offers high values for this coefficients for most beta-lactams (except cloxacillin, cefoperazone and ceftiofur), macrolides, quinolones and sulphonamides.

The high "β_1" coefficients values, which use *G. stearothermophilus* for the detection of tylosin and beta-lactam antibiotics in ovine milk, were indicated with the BRT AiM (Molina et al., 2003), Delvotest SP (Althaus et al., 2002), Charm Blue-Yellow (Linage et al., 2007) and Eclipse 100ov (Montero et al., 2005) methods. In addition, the last two methods presented high "β_1" parameters to sulfonamides. For the "QS" bioassay, Nagel (2009) indicated high "β_1" coefficients values when analyzing samples of cow's milk fortified with sulphonamides.

The concordance coefficients obtained by applying of the logistic model were high for both bioassays. They fell between 70.49 % for amoxicillin ("BT" bioassay) and 91.67 % for sulfadimethoxine ("BT" bioassay), demonstrating the correct adjustment achieved by the logistic model.

The detection capability (CCβ), calculated as concentrations which produce 95% of the positive results in dose-response curves (Codex Alimentarius, 2010), are summarized in Table 3.

As regards the beta-lactam antibiotics analyzed, the "BT" bioassay presented similar CCβ to the respective MRLs (except cefoperazone), while the "QS" bioassay detected only to penicillin residues at the MRL level. The detection capability for the "BT" bioassay for beta-lactams were similar to the values calculated for BRT AiM (amoxicillin, CCβ= 6 μg/l; ampicillin, CCβ= 6 μg/l; benzylpenicillin, CCβ= 2 μg/l; cloxacillin, CCβ= 51 μg/l; cephalexin, CCβ= 270 μg/l; cefoperazone, CCβ= 92 μg/l and ceftiofur, CCβ= 120 μg/l) for Molina et al. (2003), Eclipse 100ov (amoxicillin, CCβ= 7 μg/l, benzylpenicillin, CCβ= 5 μg/l; cloxacillin, CCβ= 68 μg/l; cephalexin, CCβ= 115 μg/l and cefoperazone, CCβ= 110 μg/l) for Montero et al. (2005), and Charm Blue-Yellow (ampicillin, CCβ= 5-6 μg/l; benzylpenicillin, CCβ= 3-4 μg/l; cloxacillin, CCβ= 33-42 μg/l; cephalexin, CCβ= 160-202 μg/l; cefoperazone, CCβ= 73-82 μg/l and ceftiofur, CCβ= 96-107 μg/l) for Linage et al. (2007), which also used *G. stearothermophilus* as the bacteria test. However, Althaus et al. (2002) indicated lower detection capability when

using the Delvotest SP test with ovine milk samples (amoxicillin, CCβ= 3 μg/l; ampicillin, CCβ= 2 μg/l; benzylpenicillin, CCβ= 1 μg/l; cloxacillin, CCβ= 18 μg/l; cephalexin, CCβ= 40 μg/l; cefoperazone, CCβ= 20 μg/l and ceftiofur, CCβ= 33 μg/l).

Table 2. Summary of logistic regression model parameters of antibiotics in ovine milk for microbiological system

Antibiotics	"BT" Bioassay			"QS" Bioassay		
	β_0	β_1	C	β_0	β_1	C
Beta-lactams						
Amoxicillin	-4,950	2,123	70,49	-4,508	1,324	73,79
Ampicillin	-5,652	2,424	74,63	-6,055	0,723	88,29
Benzylpenicillin	-10,975	5,270	77,43	-10,320	3,707	71,34
Cloxacillin	-4,771	0,308	75,47	-5,406	0,036	66,95
Oxacillin	-3,064	0,402	73,02	-5,870	0,133	86,30
Cephalexin	-3,237	0,048	79,14	-8,196	0,079	75,82
Cefoperazone	-11,619	0,084	75,72	-9,332	0,046	75,71
Ceftiofur	-11,421	0,125	88,82	-5,722	0,026	75,54
Aminoglycosides						
Gentamicin	-7,959	0,024	74,06	-14,330	0,026	77,02
Neomycin	-6,143	0,007	79,30	-16,381	0,003	78,27
Streptomycin	-8,749	0,002	86,32	-11,179	0,003	81,25
Macrolides						
Erythromycin	-9,732	0,056	78,86	-13,493	0,289	78,23
Lincomycin	-11,560	0,044	74,27	-12,445	0,055	78,87
Tylosin	-7,572	0,104	76,69	-132,074	0,951	89,08
Spiramycin	-8,380	0,003	77,42	-10,915	0,036	86,38
Quinolones						
Ciprofloxacin	-8,679	0,005	87,03	-22,162	0,152	88,26
Enrofloxacin	-9,809	0,005	86,33	-13,963	0,071	86,56
Marbofloxacin	-11,628	0,003	87,17	-11,672	0,051	75,76
Sulphonamides						
Sulfadiazine	-4,956	0,000	84,56	-5,850	0,056	80,64
Sulfadimethoxine	-16,157	0,001	91,67	-4,449	0,054	78,41
Sulfamerazine	-19,487	0,001	86,32	-4,494	0,065	76,74
Sulfamethazine	-20,267	0,001	92,65	-3,769	0,034	73,58
Sulfamethoxazole	-18,659	0,001	90,78	-5,183	0,066	79,15
Sulfathiazole	-20,429	0,001	89,46	-3,749	0,055	79,78
Tetracyclines						
Clortetracycline	-8,730	0,043	85,6	-9.254	0.026	82.4
Oxytetracycline	-6,611	0,074	72,65	-9,827	0,022	72,38
Tetracycline	-6,081	0,058	70,55	-8,053	0,013	76,67

β_0, β_1 = coefficients estimated for the logistic regression models; C: percentage concordance coefficients

Of the three aminoglycosides analyzed, only neomycin residues were detected by the "BT" bioassay at levels close to the MRL (1,500 μg/l), while gentamicin must be present at higher concentrations (450 μg/l) to be detected by this bioassay. Neither bioassay was able to detect streptomycin residues (5,000 μg/l for "BT" bioassay and 4,500 μg/l for "SQ" bioassay). It is necessary to emphasize that the BRT AiM (630 μg/l

of neomycin, 3,700 μg/l of Gentamicin and 6,000 μg/l of streptomycin), Delvotest SP (2,600 μg/l of neomycin, 1,200 μg/l of gentamicin and 6,100 μg/l of streptomycin), Eclipse 100ov (9,100 μg/l of neomycin, 3,140 μg/l of gentamicin and 10,100 μg/l of streptomycin) and Charm Blue-Yellow (444-542 μg/l of neomycin, 355-382 μg/l of gentamicin and 3,063-3,593 μg/l of streptomycin) methods obtained appropriate detection capability for neomycin (except Eclipse 100ov), high ones for gentamicin, but proved inadequate for streptomycin in ovine milk according to Althaus et al. (2002), Linage et al. (2007), Molina et al. (2003) and Montero et al. (2005), respectively.

For macrolides, Table 3 shows that the CCβ for the "QS" bioassay for erythromycin (60 μg/l), lincomycin (280 μg/l), tylosin (140 μg/l) and spiramycin (380 μg/l) were slightly above their respective MRLs, indicating good sensitivity for *B. subtilis* for that family of antibiotics in milk. On the contrary, "BT" bioassay presents a detection capability for tylosin (100 μg/l) closer to their MRL (50 μg/l) if compared to the "QS" bioassay. The low sensitivity of *G. stearothermophilus* to detect erythromycin (630 μg/l for BRT AiM, 830 μg/l for Delvotest SP, 750 μg/l for Eclipse 100ov, and 444-522 μg/l for Charm Blue-Yellow) and spiramycin (18,100 μg/l for Eclipse 100ov, and 1,106-1,346 μg/l for Charm Blue-Yellow) was pointed out by those authors.

Of the three quinolones tested, ciprofloxacin (160 μg/l) and enrofloxacin (230 μg/l) were detected by the "QS" bioassay at levels near their MRL (100 μg/l), while marbofloxacin residues must be present in milk at a higher level (280 μg/l) than the MRL (75 μg/l) to be detected by this method. In contrast, the "BT" bioassay was not sensitive to these antibiotics because it presented high CCβ for ciprofloxacin (2,280 μg/l), enrofloxacin (2,770 μg/l) and marbofloxacin (5,540 μg/l) in ovine milk. It is noteworthy that Montero, Althaus et al. (2005) reported high CCβ for ciprofloxacin (5,100 μg/l) and enrofloxacin (4,000 μg/l) when using the Eclipse 100ov method to analyze ovine milk samples fortified with quinolones. Similarly, Linage et al. (2007) reported a wide range (41,000-46,000 μg/l) for the enrofloxacin residues analyzed by the Charm Blue-Yellow method.

Once again, these studies indicate that the use of these commercial methods containing *G. stearothermophilus* is inadequate to control quinolones residues in ovine milk, and that the use of another bacteria test (i.e. *B. subtilis*) is necessary.

Table 3. Microbiological system detection capability (μg/l) for antibiotics in milk

Antibiotics	"BT" Bioassay [a]	"QS" Bioassay [a]	MRL[b]
Beta-lactams			
Amoxicillin	4	6	4
Ampicillin	4	12	4
Benzylpenicillin	3	4	4
Cloxacillin	25	232	30
Oxacillin	15	66	30
Cephalexin	128	141	100
Cefoperazone	174	266	50
Ceftiofur	115	328	100
Aminoglycosides			
Gentamicin	450	670	100
Neomycin	1,360	6,700	1,500
Streptomycin	5,000	4,500	200
Macrolides			
Erythromycin	230	60	40
Lincomycin	330	280	150
Tylosin	100	140	50
Spiramycin	4,280	380	200
Quinolones			
Ciprofloxacin	2,280	160	100
Enrofloxacin	2,770	230	100
Marbofloxacin	5,540	280	75
Sulphonamides			
Sulfadiazine	53,000	157	100
Sulfadimethoxine	1300	136	100
Sulfamerazine	23,000	115	100
Sulfamethazine	35,000	200	100
Sulfamethoxazole	17,000	123	100
Sulfathiazole	17,000	122	100
Tetracyclines			
Clortetracycline	271	470	100
Oxytetracycline	129	570	100
Tetracycline	154	840	100

[a] Detection capabilities estimated as concentrations at which 95 % of the positive results
[b] MRLs (μg/l)

Regarding sulphonamides, Table 3 shows that the "QS" bioassay presented similar detection capability (sulfadiazine, CCβ= 157 μg/l; sulfadimethoxine, CCβ= 136 μg/l; sulfamerazine, CCβ= 115 μg/l; sulfamethazine, CCβ= 200 μg/l; sulfamethoxazole, CCβ= 123 μg/l and sulfathiazole, CCβ= 122 μg/l) to the MRLs. However, the "BT" Bioassay did not provide good limits for this family of antibiotics because there was no trimethoprim in the culture medium (Nagel et al., 2009).

These limits were similar to those reported for the Charm Blue-Yellow test (sulfadimethoxine, CCβ= 101-119 μg/l; sulfamethazine, CCβ= 309-328 μg/l; sulfathiazole, CCβ= 122-151 μg/l) by Linage et al. (2007), but were lower than the

levels obtained for Eclipse 100ov (sulfadimethoxine, CCβ= 170 μg/l; sulfamethazine, CCβ= 750 μg/l, and sulfathiazole, CCβ= 250 μg/l) reported by Montero et al. (2005) when using *G. stearothemophilus* instead of *B. subtilis*. Nevertheless, Althaus et al. (2002) calculated lower detection capability (sulfadiazine, CCβ= 88 μg/l and sulfamethoxazole, CCβ= 44 μg/l) than those obtained in this work (Table 3) when analyzing ovine milk samples by the Delvotest SP method.

To synthesize, the Figure 1 shows the detection pattern by the simultaneous implementation of "BT" and "QS" bioassays. This scale was constructed by applying the logarithmic transformation to CCβ/MRL for each antimicrobial. The interior, central and outer polygons correspond to concentrations equivalent to 10xMRL, MRL, and 0.1xMRL, respectively.
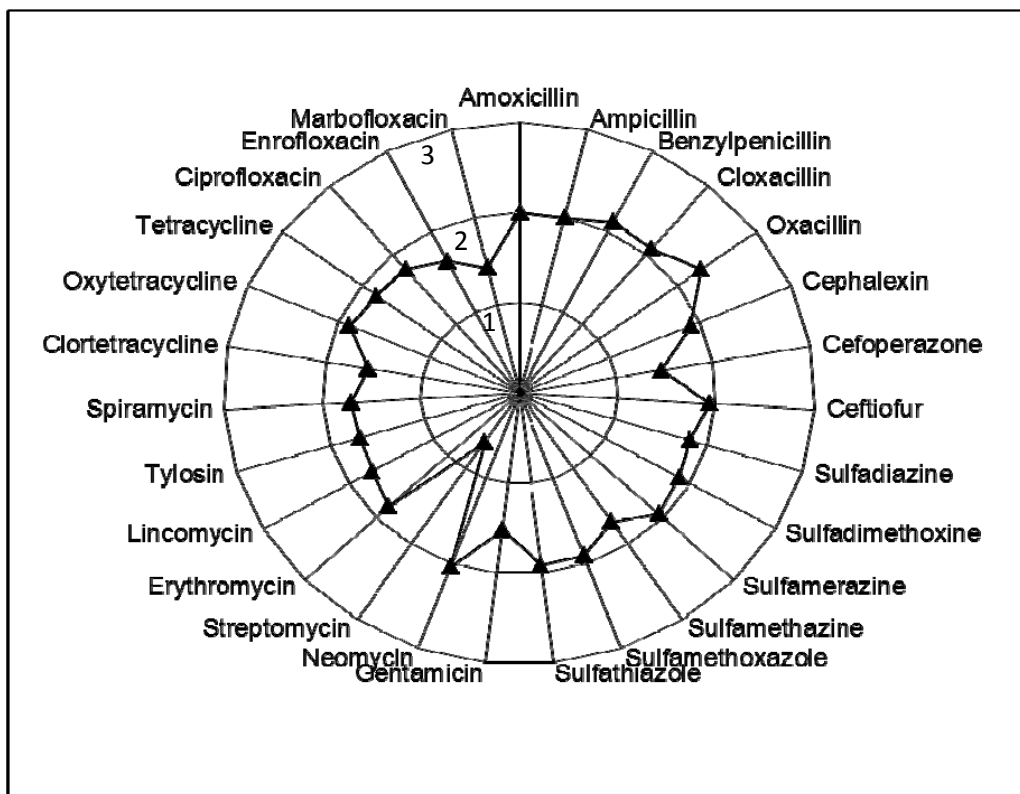
Figure 1. Detection pattern by simultaneous implementation of "BT" and "QS" bioassays. Line 1: 10xCCβ/MRL; Line 2: CCβ/MRL and Line 3: 0.1xCCβ/MRL
(Note: The figure uses the lowest CCβ of antibiotics listed in Table 3)

This figure summarizes the adequate detection capability of the microbiological system, since most of the antibiotics have detection capability near their corresponding MRLs, with the exception of streptomycin. It is noted that the CCβ of the different antibiotics analyzed by this microbiological system are located close to central polygon (MRL).

4. Conclusions

The microbiological system consists of two bioassays using *G. stearothermophilus* and *B. Subtilis*, which can detect a large number of antibiotics in milk (beta-lactams, quinolones, sulphonamides, tetracyclines, erythromycin, lincomycin, neomycin, spiramycin and tylosin) if compared with other currently used microbiological methods.

This improved detection of antibiotic residues is achieved by using two bacteria tests with complementary sensitivity to detect different antibiotics.

Therefore, this microbiological system proves to be a valuable tool to control the quality of ovine milk. The implementation of this system with two bacteria tests enables a more rigorous control of antibiotic residues in milk and, consequently, helps protect consumers' health.

5. Acknowledgments

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Chapter 4

Evaluation of the receptor-binding assays for screening antibiotics in sheep and goat's milk

Evaluation of the Charm MRL BLTET test for the detection of antibiotics in sheep and goat's milk

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

[a]Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera, s/n. 46071. Valencia, Spain.

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

Abstract

The Charm MRL Beta-Lactam and Tetracycline test (Charm MRL BLTET test. Charm Sciences Inc., Lawrence, MA) is an immunoreceptor assay utilizing ROSA® (Rapid One Step Assay) lateral flow technology that detects beta-lactam and/or tetracycline drugs in raw commingled cow milk at or below EU-MRLs. The Charm MRL BLTET test procedure was recently modified (dilution in buffer and longer incubation) by the manufacturers to be used with raw ewe`s and goat's milk. In order to assess the Charm MRL BLTET test for the detection of beta-lactams and tetracyclines in milk of small ruminants, an evaluation study was performed at Instituto de Ciencia y Tecnologia Animal (ICTA) of Universitat Politècnica de València (Spain). The test specificity and detection capability (CCβ) were studied following Commission Decision 2002/657/EC. Specificity results obtained in this study were optimal for individual milk free of antimicrobials from ewes (99.2 % for beta-lactams and 100 % for tetracyclines) and goats (97.9 % for beta-lactams and 100 % for tetracyclines) along the entire lactation period regardless of whether the results were visually or instrumentally interpreted. Moreover, no positive results were obtained when a relatively high concentration of different substances belonging to antimicrobial families other than beta-lactams and tetracyclines were present in ewe's and goat's milk. For both types of milk, the CCβ calculated was lower or equal to EU-MRL for amoxicillin (4 μg/Kg), ampicillin (4 μg/Kg), benzylpenicillin (≤ 2 μg/Kg), dicloxacillin (30 μg/Kg), oxacillin (30 μg/Kg), cefacetrile (≤ 63 μg/Kg), cefalonium (≤ 10 μg/Kg), cefapirin (≤ 30 μg/Kg), desacetylcefapirin (≤ 30 μg/Kg), cefazolin (≤ 25 μg/Kg), cefoperazone (≤ 25 μg/Kg), cefquinome (20 μg/Kg), ceftiofur (≤ 50 μg/Kg), desfuroylceftiofur (≤ 50 μg/Kg) and cephalexin (≤ 50 μg/Kg). However, this test could neither detect cloxacillin nor nafcillin at or below EU-MRL (CCβ > 30 μg/Kg). The CCβ for tetracyclines was also lower than EU-MRL for chlortetracycline (ewe's milk: ≤ 50 μg/Kg and goat's milk: 75 μg/Kg), oxytetracycline (≤ 50 μg/Kg) and tetracycline (≤ 50 μg/Kg). Regarding the 4-epimers of these tetracyclines only 4-epioxytetracycline was detected by the Charm MRL BLTET test below EU-MRL (ewe's milk: 75 μg/Kg and goat's milk: ≤ 50 μg/Kg). Azidiol had no effect on the performance of the test. The Charm MRL BLTET test could be used routinely with adapted test procedure for the fast screening of ewe's and goat's milk.

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1. Introduction

In dairy sheep and goats, just as in dairy cows, treatment of mastitis and other infectious diseases with pharmacological products is a standard practice. In many cases, antibiotic milk contamination may be caused by treatments carried out without a veterinary prescription and with inadequate knowledge of the suitable dosage, administration route or depletion time of the antibiotic substance (Molina et al., 2003a). This is partly due to the fact that there are very few drugs on the market specifically authorised for the use in lactating small ruminants, particularly goats, and occasionally veterinarians can prescribe drugs under 'cascade'. Due to inter-species differences, available bovine data cannot be accurately extrapolated for the use in the dairy ewes and goats (Pengor and Kirbis, 2009).

Drug residues in milk supplies may not only have public health implications (Phillips et al., 2004; Sanders et al., 2011) but may also interfere in the manufacture of dairy products such as cheeses and yoghurts (Packham et al., 2001; Berruga et al., 2011).

In some Mediterranean countries such as Spain, France, Italy and Greece, the production of ewe's and goat's milk plays a prominent role because of tradition and successful commercialization into products such as different cheeses and yoghurt (Haenlein, 2001). For this reason, milk quality is mainly evaluated in terms of its technological or coagulation properties which can be affected by the presence of antibiotic residues in milk.

To avoid risks related to drug residues, the control of the presence of veterinary medicinal products in foodstuffs of animal origin at different stages of the production process is legally binding in many countries. The US Food and Drug Administration Center for Veterinary Medicine (FDA) established Safe Levels/Tolerance of antibiotic residues in milk for the consumer protection (FDA, 2005). In the European Union, the regulatory levels or maximum residue limits (EU-MRLs) are defined by Regulation (EC) 470/2009 (European Union, 2009) and established by Commission Regulation (EU) 37/2010 (European Union, 2010).

Currently, numerous screening tests are commercially available to detect all kinds of antibiotics in milk (IDF, 2010). Choosing a test depends on the control step (farms, dairies or laboratories) and on the antibiotics used in the area of milk production. In farms and dairies, receptor binding assays are most commonly applied due to their simple and fast response. These methods, based on the use of specific receptors to detect antibiotics, were originally designed for the swift detection of beta-

lactam antibiotics in cow's milk (Charm and Zomer, 1995). Along recent years these tests have been further developed, and there are currently specific receptor binding assays available for the detection of various antimicrobials such as tetracyclines, gentamicin, enrofloxacin or sulfonamides. Improvements made have also been directed at the reduction of the analysis period required and the inclusion of different receptors in one test type, having resulted in combined tests capable of detecting various groups of antibiotics simultaneously.

The Charm MRL Beta-Lactam and Tetracycline test (Charm Sciences Inc., Lawrence, MA) is an immunoreceptor assay utilizing ROSA® (Rapid One Step Assay) lateral flow technology that detects beta-lactam and/or tetracycline drugs in raw commingled cow milk at or below EU-MRLs. This test is widely used for screening cow's milk, and the test procedure was recently modified by the manufacturers to be used with raw milk from ewes and goats.

In order to assess the Charm MRL BLTET test for the detection of beta-lactams and tetracyclines in milk of small ruminants, an evaluation study was performed at Instituto de Ciencia y Tecnologia Animal (ICTA) of Universitat Politècnica de València (Spain). The test specificity and detection capability (CCβ) were studied following Commission Decision 2002/657/EC (European Union, 2002).

2. Materials and methods

2.1. Milk samples

In order to obtain antibiotic-free milk samples along the entire lactation period, the experimental flocks of Manchega ewes of Universidad de Castilla-La Mancha (Albacete, Spain) and Murciano-Granadina goats of Universitat Politècnica de València (Valencia, Spain) were used. Animals had a good health status and did not receive any veterinary treatment neither before nor during the experimental period.

2.2. Test specificity.

Commission Decision 2002/657/EC (European Union, 2002) describes specificity as the ability of a method to distinguish between the analyte being measured and other related substances including the matrix constituents. According to this EC Regulation specificity for the Charm MRL BLTET test was investigated using two approaches: the false-positive rate was calculated when antibiotic-free milk samples were analyzed, and the study of possible interferences related to the presence of substances belonging to

antimicrobial families other than beta-lactams and tetracyclines in milk samples (cross-reaction) was carried out.

To calculate the false-positive rate of the Charm MRL BLTET test individual milk samples (200 ml) from 25 ewes and 25 goats were collected fortnightly along the entire lactation period. Ewe's milk samples were obtained at the morning milking from the first week after weaning until the end of lactation (5 months). Goat's milk was collected from the second week postpartum during a period of seven months.

Milk samples were analyzed using MilkoScan 6000 (Foss, Hiller∅d, Denmark) to determine their chemical composition (fat, protein and total solids); SCC (somatic cell count) was obtained using Fossomatic 5000 (Foss, Hiller∅d, Denmark); BC (bacterial count) was determined using Bactoscan FC (Foss, Hiller∅d, Denmark) and the pH value was measured by a conventional pHmeter (Crison, Barcelona, Spain).

Antibiotic-free milk samples (n= 250 for ewes and n= 350 for goats) were tested employing the Charm MRL BLTET test to assess the test specificity with each species. Samples giving positive results were retested (three replicates). Only samples showing positive results in at least two replicate analyses were classified as positive. Specificity was calculated as the percentage of negative samples with respect to the total of samples analyzed.

To check for interferences related to antimicrobial substances other than beta-lactams and tetracyclines (cross-reaction), 20 individual raw milk samples free of antimicrobials, 10 for ewes and 10 for goats, were collected in the mid-lactation period. Milk samples were spiked individually with a relatively high concentration of different drugs and analyzed by Charm MRL BLTET test. In agreement with Reybroeck et al. (2010), the drug concentration in milk samples was 10xEU-MRL, and one substance was chosen from each of the most important groups of antimicrobials: neomycin (aminoglycosides), lincomycin (lincosamides), erythromycin (macrolides), colistin (polimyxins), enrofloxacin (quinolones) and sulfadiazine (sulfonamides).

2.3. Detection Capability (CCβ).

The International Dairy Federation (IDF, 2002) establishes the requirements for the milk samples selected for use as "negative milk" in the evaluation studies of screening tests for antibiotics detection. These requirements have been established only for cow's milk. However, if a test is applied for milk of an animal species other than cows, the requirements with respect to the status of the animal should be adjusted accordingly.

Individual milk samples (200 ml) were collected in the mid-lactation period from 40 ewes (more than 60 days and below 90 days postpartum) and 40 goats (more than 90 days and below 150 days postpartum). The samples were refrigerated at 4 ºC and were analyzed to determine their pH, chemical composition and hygienic quality within 24 h after milking, using the analytical methods mentioned previously. For Manchega ewes' milk, fat content was between 5 % and 9 %, protein between 4.7 % and 8 % and total solids between 15 % and 22 %. Concerning hygienic quality, somatic cell count was < 300×10^3 cell/ml and bacterial count was < 10^5 cfu/ml. The pH value for ewe's milk samples was between 6.6 and 6.8. For milk from Murciano-Granadina goats, fat content was between 3.3 % and 7 %, protein between 3.1 % and 4.7 %, and total solids between 12 % and 17 %. Somatic cell count was < 750×10^3 cell/ml, and bacterial count was < 10^5 cfu/ml. The pH value for goats' milk was between 6.5 and 6.8.

Selected antibiotic-free milk samples were analyzed by the Charm MRL BLTET test, and the samples giving negative results were spiked with different beta-lactams and tetracyclines to calculate the detection capability (CCβ) of this test.

Detection capability (CCβ) was calculated according to the "Guidelines for the validation of screening methods for residues of veterinary medicines" proposed for Community Reference Laboratories Residues (CRLs, 2010). This guideline document supplements Commission Decision 2002/657/EC, and defines CCβ as the concentration at which only ≤ 5 % false compliant results remain. For authorized analytes, the concentration at which a screening test categorizes the sample as "screen positive" (potentially non-compliant) and triggers a confirmatory test is called Screening Target Concentration (STC) and it must be at or below EU-MRL. If the STC is set at half EU-MRL, the occurrence of one or no false-compliant results following the analysis of at least 20 "screen positive" control samples is sufficient to demonstrate that CCβ is below EU-MRL and below or equal to 50 % of EU-MRL. If STC is set between 50 % and 90 % of EU-MRL, at least 40 "screen positive" control samples with no more than 2 false-non compliant results will be sufficient to demonstrate that CCβ is below EU-MRL. If STC approaches EU-MRL (below 10 % of EU-MRL) a maximum of 60 replicates with no more than 3 false-non compliant results is required to demonstrate that CCβ is fit for this purpose. Antibiotic concentrations used for the calculation of the CCβ of the Charm MRL BLTET test were initially 0.5xEU-MRL (20 replicates); 0.75xEU-MRL (40 replicates) and 1xEU-MRL (60 replicates), respectively, only when necessary.

2.4. Effect of preservative azidiol.

To evaluate the effect of the preservative azidiol on the response of the Charm MRL BLTET test, antibiotic-free milk samples from 25 ewes and 25 goats were used. Individual milk samples were divided into two aliquots; one without preservative and one with azidiol; and analyzed by the Charm MRL BLTET test. Thereafter, each milk sample was spiked with benzylpenicillin and oxytetracycline at EU-MRL (4 µg/Kg and 100 µg/Kg, respectively) and analyzed again by the Charm MRL BLTET test.

Azidiol was prepared and used according to the Spanish regulation (Real Decreto 752/2011) which stipulates the composition (0.75 g chloramphenicol, 10 ml ethanol, 18 g sodium azide, 45 g trisodium citrate $5.5H_2O$, 0.35 g bromophenol blue, in 1000 ml of distilled water) and the dosage of this preservative in ewe's and goat's milk (133 µl per 40 ml of raw milk).

2.5. Antibiotics and spiked milk samples

The antibiotics used in this study were stored and handled according to the manufacturer's instructions before use.

Drugs were dissolved (1mg/ml) in water in a 25 ml volumetric flask at the time when analyses were carried out. In some cases the use of a small amount of a suitable solvent was necessary before adding water. Table 1 summarizes antibiotic commercial references and the solvent employed for the preparation of antibiotic stock solutions.

Spiked milk samples were prepared following the recommendations of the International Dairy Federation (IDF, 2002) and milk analysis was performed within four hours after spiking.

2.6. Test procedure

The Charm MRL BLTET test (Charm Sciences, Inc., Lawrence, MA) was employed following the manufacturer's instructions. For ewes and goats, 300 µl of milk sample was mixed with 300 µl of the dilution buffer (Sheep milk dilution buffer or Goat milk dilution buffer, respectively. Charm Sciences, Inc.) and refrigerated for 10 minutes. Thereafter, 300 µl of the mixture were placed in the sample compartment of the strip placed in the ROSA Incubator (Charm Sciences, Inc.). The incubation time was set at 56 ºC for 16 minutes (two sets of 8 minutes), and results were interpreted visually by three trained laboratory technicians and with the ROSA® Reader (ROSA® Pearl Reader. Charm Sciences, Inc.).

The Charm MRL BLTET test uses receptors that bind beta-lactam and tetracycline drugs. As milk flows through the test strip, unreacted receptors bind at the BL and/or TET position and form a visible reddish test line. A weaker intensity BL or TET line forms when beta-lactam and/or tetracycline drugs are present in the milk sample.

Table 1. Antimicrobials used to evaluate the Charm MRL BLTET test in ewe's and goat's milk

Antimicrobials	Distributor	Commercial reference	Solvent
Aminoglycosids			
Neomycin	Sigma-Aldrich[1]	N1876	H_2O
Beta-lactams			
Amoxicillin	Sigma-Aldrich	A8523	H_2O
Ampicillin	Sigma-Aldrich	A9518	H_2O
Benzylpenicillin	Sigma-Aldrich	PENNA	H_2O
Cloxacillin	Sigma-Aldrich	C9393	H_2O
Dicloxacillin	Sigma-Aldrich	D9016	MeOH / H_2O
Nafcilin	Sigma-Aldrich	N3269	MeOH / H_2O
Oxacillin	Sigma-Aldrich	46589	MeOH / H_2O
Cefacetrile	Fatro[2]	*	H_2O
Cefalonium	Sigma-Aldrich	32904	NaOH 0.1N /H_2O
Cefapirin	Sigma-Aldrich	43989	H_2O
Desacetylcefapirin	ACS Dobfar[3]	*	H_2O
Cefazolin	Sigma-Aldrich	C5020	H_2O
Cefoperazone	Sigma-Aldrich	32426	NaOH 1N / H_2O
Cefquinome	Sigma-Aldrich	32472	H_2O
Ceftiofur	Sigma-Aldrich	34001	NaOH 0.1N / H_2O
Desfuroylceftiofur	TRC[4]	D289980	MeOH / H_2O
Cephalexin	Sigma-Aldrich	C4895	H_2O
Lincosamides			
Lincomycin	Sigma-Aldrich	31727	H_2O
Macrolides			
Erythromycin	Sigma-Aldrich	E6376	EtOH / H_2O
Polimyxins			
Colistin	Sigma-Aldrich	C4461	H_2O
Quinolones			
Enrofloxacin	Sigma-Aldrich	33699	AcOH 5% / H_2O
Sulfonamides			
Sulfadiazine	Sigma-Aldrich	S6387	H_2O
Tetracyclines			
Chlortetracycline	Sigma-Aldrich	C4881	NaOH 0.1N / H_2O
4-epichlortetracycline	Acros[5]	268235000	MeOH / H_2O
Oxytetracycline	Sigma-Aldrich	O4636	HCl 0.1N / H_2O
4-epioxytetracycline	Acros	25771	MeOH / H_2O
Tetracycline	Sigma-Aldrich	T3258	HCl 0.1N / H_2O
4-epitetracycline	Acros	233125000	MeOH / H_2O

[1]Sigma-Aldrich Química, S.A. (Madrid, Spain)
[2]Fatro, S.p.A. (Bologna, Italy)
[3]ACS Dobfar, S.p.A. (Milan, Italy)
[4]Toronto Research Chemicals, Inc. (Toronto, Canada)
[5]Acros Organics (Geel, Belgium)
*Commercial reference not available

The visual interpretation of the results was carried out by comparing the BL and TET lines with the C (control) line. If both lines are darker than or equal to the C line, the milk sample is negative (antibiotic-free). If either the BL or TET line is lighter than the C line or the BL or TET line does not form, the sample is positive (likely antibiotic presence).

The performance of the reader system was checked daily by low and high calibration strips and by testing negative and positive control standards (benzylpenicillin: 4 µg/Kg and oxytetracycline: 100 µg/Kg; Charm MRL BLTET Positive tablet. Charm Sciences, Inc.) prior to testing samples. Milk samples giving a reader value ≤ 0 were considered negative, while milk samples giving a reader value > 0 were considered positive.

2.7. Statistical analysis

To assess the effect of the reading system used for the interpretation of the test results (visual or instrumental) on the test response, a chi-square test was employed. When an expected frequency was < 5 the Fisher's exact test was applied. A significant difference was defined by $p < 0.05$. Statistical analysis was performed using SAS (version 9.2, 2001; SAS Institute, Inc., Cary, NC).

3. Results and discussion

3.1. Test Specificity

Table 2 summarizes the chemical composition and hygienic quality of the individual milk samples used to assess the false-positive rate of the Charm MRL BLTET test. Mean milk sample quality parameters were similar to those reported by other authors for ewe's (Requena et al., 2010) and goat's milk (Salama et al., 2003).

Table 2. Quality parameters of ewe's and goat's milk samples obtained along the entire lactation period

Parameter	Ewe's milk (n= 250)				Goat's milk (n= 350)			
	Average	SD[1]	Min[2]	Max[3]	Average	SD[1]	Min[2]	Max[3]
pH	6.67	0.08	6.52	6.92	6.78	0.09	6.55	7.13
Fat (%)	6.38	1.94	2.42	12.68	5.74	1.16	3.31	10.61
Protein (%)	5.81	0.72	4.55	7.82	3.82	0.48	2.68	6.03
Total solids (%)	18.02	2.54	12.51	26.53	15.0	1.51	12.13	20.48
BC[4] (x10³ cfu/ml)	566	1,508	6	9,999	74	306	10	4,829
SCC[5] (x10³ cell/ml)	687	2,667	10	20,581	975	1,737	37	16,837

[1] SD: standard deviation; [2] Min: minimum; [3] Max: maximum; [4] BC: bacterial count; [5] SCC: somatic cell count

According to the instrumental interpretation (Table 3), specificity of the Charm MRL BLTET test with adapted assay procedure for the detection of beta-lactam antibiotics (BL line) was 99.2 % for ewes' milk (a false-positive rate of 0.8 %) and 97.9 % for goats' milk (a false-positive rate of 2.1 %). Specificity was 100 % for the detection of tetracyclines (TET line) in ewes' and goats' milk (no false-positive results). In all cases, the specificity calculated according to the visual interpretation of the results was slightly lower than that obtained by the ROSA® Reader, but no statistically significant differences were found (p > 0.05).

Table 3. Specificity (false-positive rate) of the Charm MRL BLTET test in antibiotic-free milk from ewes and goats with adapted test procedure

Milk samples	Test line	Results						
		Visual				Instrumental		
		P	Q	N	S (%)	P	N	S (%)
Ewes (n = 250)	BL	2	1	247	98.8	2	248	99.2
	TET	0	0	250	100	0	250	100
Goats (n = 350)	BL	7	2	341	97.4	7	343	97.9
	TET	0	1	349	99.7	0	350	100

P: positive, Q: questionable, N: negative, S (%): Specificity = negatives/total x 100

Specificity results obtained in this study were optimal for both types of milk and indicate that the characteristics of the milk do not influence the test response. The few goat's milk samples that were classified as positive (7 false-positive results) had standard characteristics of the Murciano-Granadina breed. The mean values for the quality parameters considered were: pH: 6.73, fat: 6.47 %, protein: 4.12 %, total solids: 16.04 %, SCC: 519×10^3 cell/ml and BC: 62×10^3 cfu/ml.

There is only a limited number of evaluation studies of receptor binding assays in ewe's and goat's milk available. Reybroeck et al. (2010) for the Betastar (1+1) test (Neogen Corporation, Lansing, MI) obtained a specificity of 96.8 % for ewes' milk (1 out of 31 antibiotic-free milk samples) and 96.5 % for goats' milk (1 out of 29). The same result (96.7 %) was obtained by Zeng et al. (1998) for the SNAP Betalactam test (IDEXX Laboratories, Westbrook, ME) using raw commingled goats' milk (1 out of 30).

Comparing our results with those reported by other authors with different receptor binding assays from Charm Sciences, Inc. (Lawrence, MA), Berruga et al. (2009) using the Charm MRL BLTET test in ewe's milk obtained a lower specificity for the detection of beta-lactam antibiotics (90 %) and a similar specificity (99 %) for tetracyclines. Although these authors also used individual ewe's milk for the evaluation of this test, it must be emphasized that they followed the same procedure

recommended for cow's milk (no buffer dilution used and incubation time at 56 ºC for 8 minutes) which could explain the differences observed.

Specificity of the Charm MRL BLTET test obtained in this study with adapted test procedure for individual goat's milk (97.4 % and 97.9 % for visual or instrumental interpretation, respectively) was similar to that found by Reybroeck et al., (2011) using the beta-lactam screening test Charm MRL-3 test (Charm Sciences, Inc.) with individual cow's milk samples (97.6 %). This low false-positive rate (between 2.1 % and 2.6 %) could be related to the use of individual milk samples, since these same authors calculated a specificity of 99.3 % when analyzing farm milk samples from cows. On the contrary, for ewes' and goats' milk a high incidence of false-positive results (10 out of 12 and 6 out of 8, respectively) was obtained, suggesting that the Charm MRL 3 test is not suitable for the detection of beta-lactam antibiotics in non-cow milk samples. Also, Salter et al. (2011), indicate for the Charm 3 SL3 β-Lactam test (Charm Sciences, Inc.) a specificity of 100 % for raw commingled milk from cows.

Regarding the cross-reaction study for the Charm MRL BLTET test, no positive results were obtained when a relatively high concentration (10xEU-MRL) of different substances belonging to antimicrobial families other than beta-lactams and tetracyclines were present in ewe's and goat's milk. These results are similar to those found by Reybroeck et al. (2011) and Salter et al. (2011) who neither found interferences due to the presence of other non-beta-lactam antimicrobials in milk from cows using the Charm MRL-3 test and Charm 3 SL3 β-Lactam test (Charm Sciences, Inc.), respectively.

3.2. Detection capability (CCβ)

Detection capability results (CCβ values) of the Charm MRL BLTET with adapted test procedure for different beta-lactams and tetracyclines in ewe's and goat's milk were evaluated. The CCβ values calculated according to the visual interpretation of the results were the same as those obtained by the ROSA® Reader and are summarized in Tables 4 and 5.

For both types of milk, the CCβ calculated was lower than EU-MRL for benzylpenicillin (≤ 2 µg/Kg), cefacetrile (≤ 63 µg/Kg), cefalonium (≤ 10 µg/Kg), cefapirin (≤ 30 µg/Kg), desacetylcefapirin (≤ 30 µg/Kg), cefazolin (≤ 25 µg/Kg), cefoperazone (≤ 25 µg/Kg), ceftiofur (≤ 50 µg/Kg), desfuroylceftiofur (≤ 50 µg/Kg) and cephalexin (≤ 50 µg/Kg). For amoxicillin (4 µg/Kg), ampicillin (4 µg/Kg), dicloxacillin (30 µg/Kg), oxacillin (30 µg/Kg) and cefquinome (20 µg/Kg) the Charm MRL BLTET CCβ was equal to EU-

MRL. However, this test could neither detect cloxacillin nor nafcillin at or below EU-MRL (CCβ > 30 µg/Kg).

Table 4. Detection capability (CCβ values) of the Charm MRL BLTET test for antibiotics in ewe's milk with adapted test procedure

Antimicrobials	EU-MRL (µg/Kg)	STC[1] (µg/Kg)	Positive/Total samples[2]	Positive Results (%)	CCβ (µg/Kg)
Beta-lactams					
Amoxicillin	4	4	57/60	95	4
Ampicillin	4	4	58/60	97	4
Benzylpenicillin	4	2	19/20	95	≤ 2
Cloxacillin	30	30	11/60	18	> 30
Dicloxacillin	30	30	57/60	95	30
Nafcilin	30	30	22/60	37	> 30
Oxacillin	30	30	59/60	98	30
Cefacetrile	125	63	20/20	100	≤ 63
Cefalonium	20	10	20/20	100	≤ 10
Cefapirin	60[3]	30	20/20	100	≤ 30
Desacetylcefapirin	*	30	20/20	100	≤ 30
Cefazolin	50	25	20/20	100	≤ 25
Cefoperazone	50	25	20/20	100	≤ 25
Cefquinome	20	20	60/60	100	20
Ceftiofur	100[4]	50	20/20	100	≤ 50
Desfuroylceftiofur	*	50	20/20	100	≤ 50
Cephalexin	100	50	20/20	100	≤ 50
Tetracyclines					
Chlortetracycline	100[5]	50	20/20	100	≤ 50
4-epichlortetracycline	*	100	0/60	0	> 100
Oxytetracycline	100[5]	50	20/20	100	≤ 50
4-epioxytetracycline	*	75	40/40	100	75
Tetracycline	100[5]	50	20/20	100	≤ 50
4-epitetracycline	*	100	0/60	0	> 100

[1]STC: Screening Target Concentration
[2]According to the CRLs (2010) STC= 0.5xEU-MRL: 20 samples; STC= 0.75xEU-MRL: 40 samples; STC= 1xEU-MRL: 60 samples
[3]sum of cefapirin and desacetylcefapirin
[4]sum of all residues retaining the beta-lactam structure expressed as desfuroylceftiofur
[5]sum of parent drug and its 4-epimer
*marker residue. EU-MRL not established

The CCβ for tetracyclines was also lower than EU-MRL for chlortetracycline (ewe's milk: ≤ 50 µg/Kg) and goat's milk: 75 µg/Kg), oxytetracycline (≤ 50 µg/Kg) and tetracycline (≤ 50 µg/Kg). Regarding the 4-epimers of these tetracyclines, only 4-epioxytetracycline was detected by the Charm MRL BLTET test below EU-MRL (ewe's milk: 75 µg/Kg and goat's milk: ≤ 50 µg/Kg). For 4-epichlortetracycline and 4-epitetracycline the CCβs were above EU-MRL (CCβ > 100 µg/Kg).

Table 5. Detection capability (CCβ values) of the Charm MRL BLTET test for antibiotics in goat's milk with adapted test procedure

Antimicrobials	EU-MRL (µg/Kg)	STC[1] (µg/Kg)	Positive/Total samples[2]	Positive Results (%)	CCβ (µg/Kg)
Beta-lactams					
Amoxicillin	4	4	57/60	95	4
Ampicillin	4	4	58/60	97	4
Benzylpenicillin	4	2	20/20	100	≤ 2
Cloxacillin	30	30	9/60	15	> 30
Dicloxacillin	30	30	58/60	97	30
Nafcillin	30	30	18/60	30	> 30
Oxacillin	30	30	60/60	100	30
Cefacetrile	125	63	20/20	100	≤ 63
Cefalonium	20	10	20/20	100	≤ 10
Cefapirin	60[3]	30	20/20	100	≤ 30
Desacetylcefapirin	*	30	20/20	100	≤ 30
Cefazolin	50	25	20/20	100	≤ 25
Cefoperazone	50	25	20/20	100	≤ 25
Cefquinome	20	20	60/60	100	20
Ceftiofur	100[4]	50	20/20	100	≤ 50
Desfuroylceftiofur	*	50	20/20	100	≤ 50
Cephalexin	100	50	20/20	100	≤ 50
Tetracyclines					
Chlortetracycline	100[5]	75	38/40	95	75
4-epichlortetracycline	*	100	0/60	0	> 100
Oxytetracycline	100[5]	50	20/20	100	≤ 50
4-epioxytetracycline	*	50	20/20	100	≤ 50
Tetracycline	100[5]	50	19/20	95	≤ 50
4-epitetracycline	*	100	8/60	13	> 100

[1]STC: Screening Target Concentration
[2]According to the CRLs (2010) STC= 0.5xEU-MRL: 20 samples; STC= 0.75xEU-MRL: 40 samples; STC= 1xEU-MRL: 60 samples
[3]sum of cefapirin and desacetylcefapirin
[4]sum of all residues retaining the beta-lactam structure expressed as desfuroylceftiofur
[5]sum of parent drug and its 4-epimer
*marker residue. EU-MRL not established

These results (CCβ ≤ EU-MRL) are similar to those obtained by Reybroeck et al. (2011) using the Charm MRL-3 test (Charm Sciences, Inc.) to detect beta-lactams in cow's milk samples; the only exception being cloxacillin which was also detected by these authors at a concentration below EU-MRL (14 µg/Kg). Salter et al. (2011) also obtained appropriate sensitivity with the Charm 3 SL3 β-lactam test (Charm Sciences, Inc.) according to Safe Level/Tolerance as stipulated by the US FDA (2005).

3.3. Effect of azidiol on the test response

The presence of azidiol in milk samples had no influence on the response of the Charm MRL BLTET test. All the antibiotic-free milk samples from ewes and goats spiked with azidiol were clearly negative (Figure 1) regardless of the system used for

the interpretation of the results. No interference was observed neither with milk samples spiked with benzylpenicillin (4 µg/Kg) nor with oxytetracycline (100 µg/Kg) no matter whether the interpretation of the results was made visually or instrumentally.
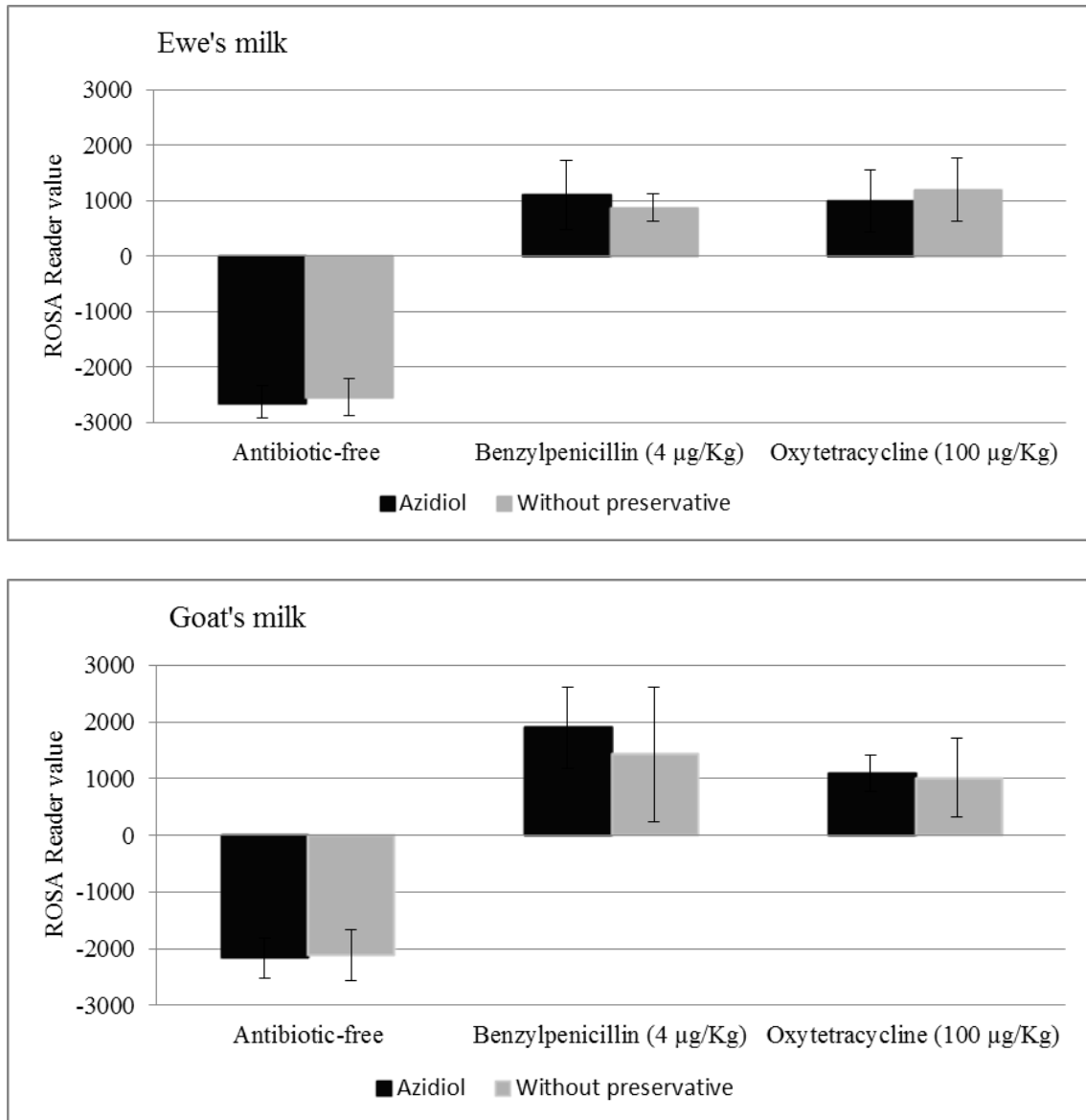
Figure 1. Effect of azidiol in ewe's and goat's milk samples on the results of the Charm MRL BLTET test

So far, there is no study on the influence of preservatives on the performance of the receptor binding assays for the detection of antibiotics in milk available. Only studies with microbial inhibitor tests have been carried out as the presence of preservatives may interfere with the growth of the microorganism in the test, increasing the incidence of questionable or false-positive results (Molina et al., 2003b).

The results obtained in this study show the suitability of the Charm MRL BLTET test for the detection of antibiotic residues of beta-lactams and tetracyclines in ewe's and goat's milk. The Charm MRL BLTET test was neither influenced by the distinct composition of ewe's and goat's milk, characterised by an elevated fat and protein contents when compared to cow's milk, nor by the high somatic cell count which some authors related to false positive results in the microbial screening tests (Althaus et al., 2003) and receptor binding assays (Contreras et al., 1997).

These results are of great relevance for ovine and caprine milk quality control programs. The Charm MRL BLTET test enables the fast and efficient control of antibiotics in farms and the dairy industry, thus guaranteeing the absence or presence below legally established EU-MRLs of most beta-lactams and tetracyclines. Moreover, the Charm MRL BLTET test was not affected by the presence of the preservative azidiol in milk samples, which also allows its use in milk quality control laboratories which normally analyze ewe's and goat's milk with azidiol.

The only aspects of the test which could possibly be improved are the test duration (16 minutes), which is relatively long when compared to other protein receptor binding tests usually applied in cow's milk (1-9 minutes), and the need to dilute the ewe's and goat's milk samples with a specific buffer before analysis. In this sense, it is worth mentioning that the manufacturers are currently working on a new version of the Charm MRL BLTET test that does no require the buffer and with a shorter incubation time taking advantage of the high specificity and adequacy of receptors used in the ROSA® Charm technology.

4. Conclusions

The Charm MRL BLTET test displays a high specificity for the detection of antibiotics in ewe's and goat's milk with adapted test procedure regardless of whether the interpretation of the results is carried out visually or instrumentally. The Detection capability (CCβ values) obtained for the Charm MRL BLTET test indicates a high sensitivity to most beta-lactam antibiotics considered except for cloxacillin and nafcillin. As for tetracyclines the Charm MRL BLTET test was also able to detect chlortetracycline, oxytetracycline, tetracycline and 4-epioxytetracycline at or below EU-MRL. Azidiol had no effect on the performance of the test.

The great performance characteristics of the Charm MRL BLTET test makes it suitable to be included in ewe's and goat's milk quality programs as a fast routine method on farms and in the dairy industries.

5. Acknowledgments

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Detection of antibiotics in sheep milk by receptor-binding assays

Beltrán M.C.[a], Althaus R.L.[b], Berruga M.I.[c], Molina A.[c], Molina M.P.[a]

[a]Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera, s/n, 46071 Valencia, Spain.

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

[c]Departamento de Ciencia y Tecnología Agroforestal, ETSIA-IDR, Universidad de Castilla-La Mancha, 02071 Albacete, Spain.

Abstract

The aim of this study was to evaluate some receptor-binding assays to detect antibiotics in sheep milk. Specificity of the Betastar Combo, SNAP and Twinsensor^{BT} tests was optimal using inhibitor-free bulk sheep milk (99-100 %), and no differences between the visual or instrumental classification were found. For individual sheep milk free of antibiotics, specificity was elevated by the Betastar Combo and SNAP tests. However, lower specificity was obtained by the Twinsensor^{BT} test, especially in the last weeks of the lactation period. Regarding cross-reactions, interferences related to drugs other than β-lactams and tetracyclines were not detected. Furthermore, the use of azidiol, as a preservative of milk, had no effect on the test. In all cases, the CCβ (Detection capability) was able to detect most β-lactams and tetracyclines at or below MRLs (Maximum Residues Limits). In conclusion, the receptor-binding tests evaluated showed a very good performance in the detection of antibiotics in sheep milk, thus being suitable for milk quality control programmes.

1. Introduction

In Mediterranean countries, sheep milk production has traditionally been destined for the manufacture of cheese, often as raw milk. Cheese quality is closely related to milk composition but also to hygienic aspects such as somatic cell count, bacteriology or presence of antibiotic residues, currently legislated by EC Regulation Nº 853/2004 (EC, 2004) and EC Regulation Nº 1662/2006 (EC, 2006).

The use of antibiotics in dairy sheep to treat mastitis and other infectious diseases is a common veterinary practice that presents a high risk of contamination of the milk supply if appropriate measures are not taken. The implications of the presence of antibiotic residues in milk as a result of veterinary treatments have been documented, including negative effects on consumer's health such as allergies or the generation of antibiotic resistance (Dewdney et al., 1991; Oliver et al., 2011), and on the manufacturing processes of fermented products (Packham et al., 2001; Berruga et al., 2008).

For the screening of milk samples for antibiotic residues, there are various methods available (ISO/IDF, 2010), to detect numerous substances above the maximum residue limits (MRLs) regulated by EC Regulation Nº 37/2010 (EC, 2010). Beta-lactam and tetracycline antibiotics are the most frequently used for the treatment of bacterial infections in livestock, as a consequence, quality control programmes are mainly focusing on the detection of these antibiotics in milk. At present, rapid screening tests based on the use of specific receptors are widely used, especially in farms and in the dairy industry where a fast response is required. Receptor-binding assays have been validated for the use in raw bulk milk from cows (Perme et al., 2010; Reybroeck et al., 2010; Salter et al., 2011), but information on the performance of these tests in sheep milk is rather limited. The chemical composition of ewe's milk differs significantly from cow's milk which could affect the response of these screening tests. Moreover, in milk quality laboratories different preservatives (e. g. bronopol, azidiol) are usually employed in milk sampling (Elizondo et al., 2007; Gonzalo et al., 2010). For milk quality control programs, Spanish regulation (BOE, 2011) established the use of azidiol as a preservative in milk sampling. Azidiol is a bacteriostatic agent containing sodium azide and chloramphenicol, among other reagents. For this reason, it has been linked with lower specificity rates when microbial screening tests to detect antibiotics in sheep milk are applied (Molina et al., 2003). However, information about the effect of the preservative azidiol on the response of the receptor-binding tests is practically non-existent.

The aim of this work was to assess the suitability of some commercial receptor-binding tests to detect beta-lactams and tetracyclines in sheep milk with azidiol and in

preservative-free sheep milk samples. The evaluated parameters were specificity (false-positive rate and cross-reactions) and detection capability (CCβ) investigated for each of the tests considered, in agreement with the EC Commission Decision 2002/657 (EC, 2002).

2. Material and Methods

2.1. Receptor-binding assays

The screening assays used were the Betastar Combo test (Neogen Corporation, Lansing, MI, USA), SNAP Betalactam test (IDEXX Laboratories, Westbrook, ME, USA), SNAP Tetracycline test (IDEXX Laboratories) and Twinsensor[BT] test (Unisensor, Liege, Belgium), which employ binding reagents and have similar reaction mechanisms. The Betastar Combo and Twinsensor[BT] tests allow for simultaneous detection of both beta-lactam and tetracycline antibiotics in milk samples, and the SNAP tests used, namely SNAP Betalactam and SNAP Tetracycline tests, are specific for beta-lactams or tetracyclines, respectively. Test procedures in general include two stages: 1) preliminary incubation of the binding reagents with the milk sample results in the interaction of the antibiotics, if present and 2) the milk solution is transferred onto an immunochromatographic medium by which a colored signal development takes place when passing the various binding positions. Specific binding reagents that do not interact with antibiotic residues during preliminary incubation are bound at the corresponding binding positions and colored lines or spots appear.

Milk samples were analysed following the test procedures given by the manufacturers. Results were classified as positive or negative both visually, by three trained technicians, and instrumentally.

For visual classification of the Betastar Combo test results, the intensity of the different red test lines was compared. If the intensity of the antibiotic test line, BL (beta-lactam) and/or TE (tetracycline), was greater than or equal to the control line, the milk sample was classified as negative. However, if the antibiotic test line was weaker than the control line, the milk sample was classified as positive. For the Twinsensor[BT] test, visual interpretation of the results was made in a similar fashion, although in that case a sample was considered positive when the intensity of the antibiotic test line (BL and/or TE) was as distinct as or lighter in color than the control line. Visual interpretation of the two SNAP test results was made as follows: a blue sample spot darker than or equal to the control spot was negative, and a sample spot lighter than the control spot was positive. For a valid test, it is necessary that the control line or control spot appears after the incubation time. If the control marker is not visible, the test is considered invalid.

For instrumental classification of the test results, specific reader systems provided by the different laboratories were employed. Thus, dipsticks or specific devices were inserted into the corresponding reader immediately after the required incubation period, and numerical data were recorded. Table 1 summarizes the available information about the reader systems employed to categorize the test results. The performance of the reader systems was checked on a daily basis by testing a negative and positive control (benzylpenicillin: 4 µg/kg and oxytetracycline: 100 µg/kg) just before milk analysis.

Table 1. Reader systems employed to categorize the receptor-binding test results as positive or negative (antibiotic-free)

Reader	Manufacturer	Cut-off	Test result classification		
			Negative	Low positive	Positive
Accuscan III	Neogen Corporation (Lansing, MI, USA)	1.0	≥ 1.0	-	< 1.0
SNAPshot	IDEXX Laboratories (Westbrook, ME, USA)	1.06	< 1.06	-	≥1.06
Readsensor	Unisensor (Liege, Belgium)	1.10	> 1.10	0.9 – 1.10	< 0.90

2.2. Antibiotics and spiked milk samples

Amoxicillin (A8523), ampicillin (A9518), benzylpenicillin (PENNA), cloxacillin (C9393), dicloxacillin (D9016), nafcillin (N3269), oxacillin (46589), cefalonium (32904), cefapirin (43989), cefazolin (C5020), cefoperazone (32426), cefquinome (32472), ceftiofur (34001), cephalexin (C4895), chlortetracycline (C4881), colistin (C4461), enrofloxacin (33699), erythromycin (E6376), lincomycin (31727), neomycin (N1876), oxytetracycline (O4636), sulfadiazine (S6387), and tetracycline (T3258) were provided by Sigma-Aldrich Química, S.A. (Madrid, Spain). Desfuroylceftiofur (D289980) was supplied by Toronto Research Chemicals, Inc. (Toronto, Canada) and 4-epichlortetracycline (268235000), 4-epioxytetracycline (25771), and 4-epitetracycline were furnished by Acros Organics (Geel, Belgium). Finally, desacetylcefapirin and cefacterile, not commercially available, were kindly provided by Fatro S.p.A. (Bologna, Italy) and ACS Dobfar, S.p.A. (Milan, Italy), respectively.

For use, antibiotics were dissolved (1 mg/ml) in water or in an appropriate solvent (AcOH 5 % for enrofloxacin; EtOH for erythromycin; MetOH for desfuroylceftiofur, nafcillin, oxacillin and the 4-epimers of tetracyclines; NaOH 0.1N for cefalonium, ceftiofur and chlortetracycline) in a 25 ml volumetric flask at the time when analyses were carried out to avoid problems related to instability.

Spiked milk samples were prepared following International Dairy Federation recommendations (ISO/IDF, 2002) and tested by the different receptor-binding tests immediately after spiking.

2.3. Test Specificity

Specificity of the receptor-binding tests was investigated by calculating the percentage of false-positive results for each of them, and also, studying the potential cross-reaction interferences related to the presence of antibiotic substances other than beta-lactams and tetracyclines in milk. The effect of the preservative azidiol was also studied.

2.3.1. False-positive results

For each screening test, the false-positive rate was calculated by analysing individual sheep's milk free of antibiotics, and bulk milk samples from commercial dairy sheep farms. Individual milk samples free of antibiotics (n= 250) were obtained from 25 sheep, during the entire lactation period, belonging to the experimental flock of Manchega breed sheep of the Universidad of Castilla-La Mancha (Spain). Milk sampling was carried out fortnightly at morning milking, from the first week after weaning (35 days post-partum) until the end of the lactation period (170 days post-partum). Bulk milk samples (n= 100) were obtained from various commercial farms with Manchega sheep in the Castilla-La Mancha region (Spain). Bulk milk samples were analysed prior to use by the microbial inhibitor test Delvotest SP-NT MCS (DSM Food Specialties, Delft, the Netherlands) to assure the absence of inhibitors in milk.

Sheep milk samples were kept at 4 ºC before analysis (no longer than 48 h). Milk composition (fat, protein, lactose and total solids) was determined by MilkoScan 6000 (Foss, Hillerød, Denmark). Somatic cell count (SCC) and bacterial count (BC) were determined with a Fossomatic 5000 (Foss) and Bactoscan FC (Foss), respectively. Milk pH was measured by a conventional pH-meter (Crison, Barcelona, Spain).

All sheep milk samples were analysed by the investigated receptor-binding assays to evaluate the false-positive rate. For both types of milk (individual or bulk sheep milk), samples giving positive results were re-tested, in three replicates, and samples showing positive results in at least two replicate analyses, were recorded finally, as positive.

For each screening test, the specificity rate was calculated as the percentage of negative results divided by the total number of samples analysed.

2.3.2. Effect of azidiol on the tests performance

Spanish legislation (BOE, 2011) establishes the use of azidiol as a preservative in milk sampling for official quality control, its composition (0.75 g chloramphenicol, 10 ml ethanol, 18 g sodium azide, 45 g trisodium citrate 5.5 H_2O, 0.35 g bromophenol blue, in

1000 ml of distilled water), and its dosage in sheep milk samples (133 µl per 40 ml of raw milk sample).

The effect of azidiol on the response of the Betastar Combo, SNAP and Twinsensor[BT] tests was evaluated as follows: individual milk samples free of antibiotics from 20 sheep in the mid-lactation period were collected, milk samples were then divided into two aliquots (one preservative-free and one with azidiol) and analysed by each of the fast screening tests, in duplicate, to assess interferences on the test specificity.

To investigate the effect of the preservative on the sensitivity of the receptor-binding tests, the same antibiotic-free milk samples with azidiol and without preservative were spiked with benzylpenicillin and oxytetracycline at their MRLs (4 µg/kg and 100 µg/kg, respectively) and re-analysed with the corresponding test.

2.3.3. Cross-reactions

To assess possible interferences related to the presence of antibiotics other than beta-lactams and tetracyclines in milk (cross-reactions), 20 antibiotic-free sheep milk samples collected in the mid-lactation period, were spiked individually with a relatively high concentration of each selected drug, and tested simultaneously by the four assays investigated. In agreement with Reybroeck et al. (2010), the drug concentration in milk samples was 10xMRL, and one substance was chosen from each of the most important groups of antibiotics: neomycin (aminoglycosides), benzylpenicillin (β-lactams), lincomycin (lincosamides), erythromycin (macrolides), colistin (polimyxins), enrofloxacin (quinolones) and sulfadiazine (sulphonamides).

2.4. Detection capability (CCβ)

CCβ of the receptor-binding tests was calculated by spiking antibiotic-free milk samples with different antibiotic substances according to the "Guidelines for the validation of screening methods for residues of veterinary medicines" proposed by Community Reference Laboratories for residues (CRLs, 2010).

The guideline document defines the CCβ as the lowest antibiotic concentration assessed which produces at least 95 % positive results (false compliant results ≤ 5 %), and establishes a calculation procedure based on two premises: antibiotic concentration tested should be at or below MRL, and the total number of milk samples to be analysed depends on their relationship with the corresponding MRL (Table 2). Following these recommendations, the lowest antibiotic concentration assessed in this study was 0.5xMRL in twenty replicates, 0.75xMRL in forty replicates, and 1xMRL in sixty replicates only when necessary.

Sheep milk samples free of antibiotics used in this study as negative milk showed a good hygienic quality and a gross composition characteristic of sheep milk, according to the International Dairy Federation recommendations (ISO/IDF, 2002).

Table 2. Guidelines for the calculation of the Detection capability (CCβ) according to Community Reference Laboratories for residues (CRLs, 2010)

STC[1] (μg.kg^{-1})	Number of replicates	False-compliants permitted ($\leq$ 5 %)	CCβ[2] (μg.kg^{-1})
STC = 0.5 MRL[3]	20	1	$\leq$ 0.5 MRL
0.5 MRL < STC $\leq$ 0.90 MRL	40	2	0.5 - 0.9 MRL
0.90 MRL > STC $\leq$ 1 MRL	60	3	> 0.9 – 1 MRL

[1]STC: Screening Target Concentration. For authorized analytes, the concentration at which a screening test categorizes the sample as "screen positive" (potentially non-compliant); [2]CCβ: Detection Capability. Antibiotic concentration at which only $\leq$ 5 % false compliant results remain; [3]MRL: Maximum Residue Limit established in EC Regulation N° 37/2010

2.5. Statistical analysis

Statistical analysis was performed using SAS (version 9.2, 2001; SAS Institute, Inc., Cary, NC, USA). To evaluate the differences between the two systems of classification of the test results (visual vs instrumental), a chi-square test was employed. When an expected frequency was < 5, Fisher's exact test was applied. A significant difference was defined by P < 0.05.

A logistic regression model was applied to investigate the effect of milk quality parameters and stage of lactation period on the response of the receptor-binding assays that showed a false-positive rate > 5 %. Statistical analysis was carried out employing the stepwise option of the logistic procedure of the SAS (SAS, 2001). Variables were analysed using the following logistic model:

$$L_{ij} = logit\ [P_i] = \beta_0 + \beta_1[SL] + \beta_2[pH] + \beta_3[F] + \beta_4[P] + \beta_5[L] + \beta_6[TS] + \beta_7[logSCC] + \beta_8[logBC] + \varepsilon_{ij}\ (1)$$

where: L_{ij} is the logistic model; $[P_i]$ is the probability for the response category (positive/negative); β_0 is the intercept; β_i are the estimate parameters for the model; [SL] is the lactation stage effect (day); [pH] is the pH effect; [F] is the fat content effect; [P] is the protein content effect; [L] is the lactose content effect; [TS] is the total solids content effect; [logSCC] is the somatic cell count effect; [logBC] is the bacterial count effect; ε_{ij} is the residual error.

3. Results and Discussion

3.1. Test Specificity

The chemical composition and hygienic quality of the sheep milk samples used in the specificity study are summarized in Table 3, being consistent with those cited by other authors for sheep's milk quality parameters (Barron et al., 2001).

Table 3. Chemical composition and hygienic quality parameters of sheep milk samples used in the specificity study (false-positive rate)

Parameter	Individual milk (n = 250)				Bulk milk (n = 100)			
	Mean	SD	Min	Max	Mean	SD	Min	Max
pH	6.66	0.08	6.54	6.92	6.68	0.06	6.57	6.89
Fat (%)	6.38	2.05	2.42	12.68	7.30	0.72	5.63	10.13
Protein (%)	5.83	0.73	4.55	7.82	5.49	0.25	5.01	6.75
Lactose (%)	5.03	0.35	3.87	5.67	4.70	0.19	4.05	5.07
Total solids (%)	18.06	2.71	12.51	26.53	17.76	0.84	15.99	23.01
Log SCC[1]	5.01	0.48	4.00	7.27	6.02	0.26	5.37	6.94
Log BC[2]	4.83	0.72	3.78	6.96	5.35	0.49	4.23	6.66

[1]Log SCC: logarithm of somatic cell count; [2]Log BC: logarithm of bacterial count

As shown in Table 4, the specificity rate for beta-lactam antibiotic detection using the Betastar Combo test was 100 % (no positive results) for individual sheep milk and also for bulk sheep milk. No significant differences were found between the visual and instrumental interpretation of the test results. Similar results were obtained by Sternesjö & Johnsson (2003) using the Betastar test (Neogen Corporation), another version of the assay for fast detection of beta-lactam residues in cow's milk. For tetracycline detection using the Betastar Combo test in individual sheep milk, the specificity rate was calculated as 98.8 % (3 non-compliant results of 250 milk samples) for either result classification system. No positive results were obtained for sheep bulk milk samples (specificity of 100 %).

The specificity rate according to the instrumental interpretation of the SNAP Betalactam test results was 96.8 % (a false-positive rate of 3.2 %) for individual sheep milk, and 99 % for sheep bulk milk samples (a false-positive rate of 1 %). When interpretation of the results was carried out visually, specificity decreased slightly for individual milk samples (95.6 %), although the differences found were not statistically significant. These results differ that those reported by Bell et al. (1995), using bulk milk from cows, who obtain a higher specificity rate for the visual interpretation of the test results (98.4 % instrumentally, and 99.4 % visually). In raw commingled milk from goats, Zeng et al. (1998) found a lower specificity rate with the instrumental method (96.7 %).

Table 4. Specificity of the Betastar Combo, SNAP, and Twinsensor[BT] tests in sheep milk

| Test | Antibiotic[2] | Specificity[1] (%) | | | |
| | | Individual sheep milk (n = 250) | | Bulk sheep milk (n = 100) | |
		Visual	Instrumental	Visual	Instrumental
Betastar Combo	BL	100	100	100	100
	TE	98.8	98.8	100	100
SNAP	BL	95.6	96.8	99	99
	TE	100	100	100	100
Twinsensor[BT]	BL	87.2	86.4	100	100
	TE	100	100	100	100

[1]Specificity = negatives results/total results x 100; [2]BL: β-lactam; TE: Tetracycline

In some cases, for the SNAP Betalactam test, an increase of the analysis time was observed, especially at the end of the lactation period, possibly due to the concentration of the main components of milk that slows down the progress of the sample on the nitrocellulose strip.

The specificity rate was optimal for individual sheep milk (100 %) for the SNAP Tetracycline test, both visually and instrumentally. Neither had any false-positive results obtained for the SNAP Tetracycline test in ovine bulk milk.

Regarding the results for the detection of beta-lactam antibiotics using the Twinsensor[BT] test, the specificity for individual milk samples was 87.2 % and 86.4 % for visual or instrumental interpretation, respectively. The differences found were not statistically significant and could be related to the different perception of the colored test lines. This low specificity rate is mainly due to the high number of positive results found in the last weeks of the milking period. Therefore, test specificity was optimal (96–100 %) until 110 days postpartum and began to decline at 125 days of lactation (specificity of 90 %), reaching the minimum values (approximately 40 %) in the late stages of lactation period. These results may be related to changes in milk composition in the last stage of lactation, as a result of the decline in milk production. A logistic regression analysis was carried out to evaluate the lactation stage as well as milk characteristic effects on the response of the Twinsensor[BT] test in an attempt to explain the high rate of false-positive results. A significant effect of the lactation stage ($P < 0.01$) and a decrease in the pH value ($P < 0.01$) on the false-positive rate of the Twinsensor[BT] test were found. The statistical results obtained suggest that the probability to obtain non-compliant results using individual sheep milk free of antibiotics is much greater at the end of the milking period ($\geq$ 125 days post-partum), especially in those milk samples having a lower pH value (Figure 1).

However, no false-positive results were obtained when bulk milk samples were analysed (specificity 100 %) regardless of whether the results were visually or instrumentally interpreted. These results are in agreement with Perme et al. (2010), who also obtained a specificity rate of 100 % using bulk cow milk.
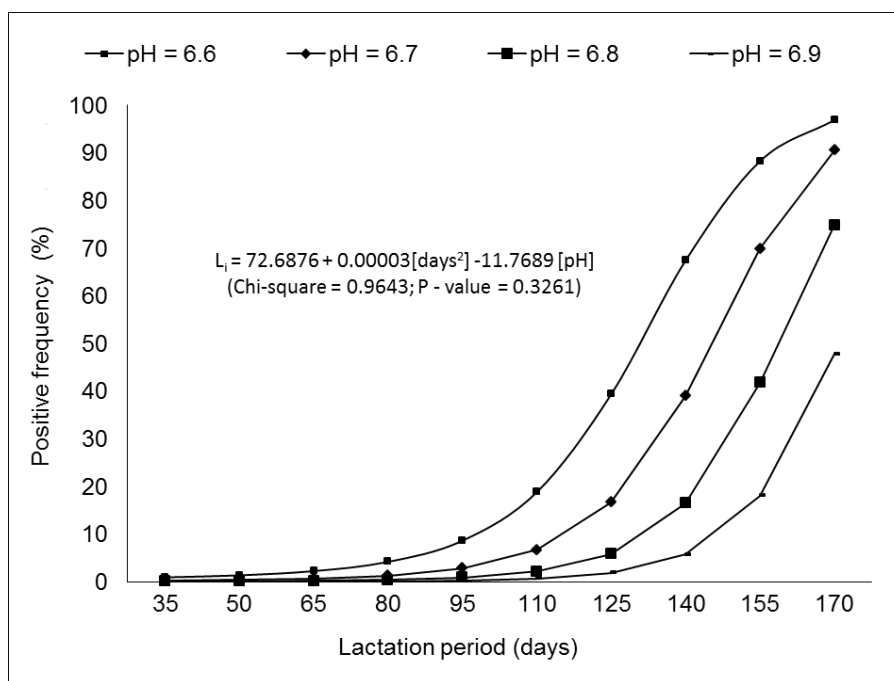
Figure1. Effect of the lactation stage and pH value of individual sheep milk samples on the specificity of the Twinsensor[BT] test

Azidiol had no effect on the performance of the receptor-binding tests assessed. No change in negative or positive results was observed, either visually or instrumentally, when antibiotic-free milk, and samples spiked with antibiotics, with and without azidiol (Table 5) were analysed. However, using the SNAP Betalactam test, a delay in the appearance of the colored spots was observed when milk with azidiol were analysed, which makes a prolonged incubation period necessary. In addition, the colored spots had a lower intensity, making the visibility of the control spot more difficult, which may in turn complicate the visual interpretation of the results.

With respect to the cross-reactivity experiment, no positive results were obtained when a relatively high concentration of different drugs other than beta-lactams and tetracyclines were present in sheep milk. These results were in accordance with those obtained by Reybroeck et al. (2010) and Salter et al. (2011) using different receptor-binding assays to detect beta-lactam antibiotics in cow milk.

Table 5. Effect of azidiol on the performance of Betastar Combo, SNAP, and Twinsensor[BT] tests

Test	Test line[1]	Antibiotic-free milk (n = 20)		Antibiotic spiked milk (n = 20)	
		NAZ[2]	AZ[3]	NAZ[2]	AZ[3]
Betastar	BL	8.494±4.15	8.863±2.65	0.003±0.00	0.008±0.00
Combo	TE	6.775±4.27	7.718±2.88	0.309±0.23	0.224±0.19
SNAP	BL	-0.77±0.15	-0.58±0.22	4.50±0.84	4.66±0.80
	TE	-0.63±0.14	-0.30±0.06	3.37±0.79	3.70±0.73
Twinsensor[BT]	BL	1.53±0.30	1.78±0.24	0.09±0.05	0.08±0.01
	TE	3.00±0.20	2.65±0.18	0.01±0.01	0.01±0.01

[1]BL: β-lactam line, TE: tetracycline line; [2]NAZ: without azidiol; [3]AZ: with azidiol

3.2. Detection capability (CCβ)

Table 6 summarizes the CCβ of the receptor-binding tests. The Betastar Combo test presents a CCβ at or below MRL for all beta-lactam antibiotics tested, except for ceftiofur, and cephalexin, being above their respective MRLs. Desfuroylceftiofur could not be detected at 100 µg/kg equivalent antibiotic concentration. For tetracyclines, the CCβ obtained by the Betastar Combo test was equal to MRL for chlortetracycline, oxytetracycline and tetracycline. However, the 4-epimers of these tetracyclines were not detected at 100 µg/kg antibiotic concentration in all cases. These results are in agreement to those reported by Reybroeck et al. (2010) using the Betastar (1+1) test (Neogen Corporation) in cow milk. Shuren and Knappstein (2004), and Žvirdauskiene and Salomskiene (2007) also indicate that the Betastar test is able to detect benzylpenicillin, amoxicillin and ampicillin at or below their respective MRLs. At the moment, there are no published studies on the CCβ of the Betastar Combo test to detect tetracyclines in milk.

As shown in Table 6, the SNAP Betalactam test was able to detect all the beta-lactam antibiotics tested at or below their respective MRLs. These results are similar to those obtained by Shuren and Reichmuth (1998) using the SNAP Betalactam test with cow milk, except for amoxicillin which could not be detected by these authors at or below MRL. Regarding tetracyclines, the CCβ of the SNAP Tetracycline test was at or below MRL for chlortetracycline, oxytetracycline, and tetracycline. However, the CCβ of this test was above 100 µg/kg for the 4-epimers considered.

The CCβ of the Twinsensor[BT] test obtained in this study was lower than MRL for all the beta-lactams tested except for cephalexin, and nafcillin. For tetracyclines, the Twinsensor[BT] test was able to detect chlortetracycline, oxytetracycline, and tetracycline at or below MRL. In contrast, this test could not detect 4-epichlortetracycline, or 4-epioxytetracycline, or 4-epitetracycline at 100 µg/kg equivalent antibiotic concentration.

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These results are in accordance to those reported by Perme et al. (2010) in their study on the performance of the Twinsensor[BT] test in cow milk, who could not detect nafcillin or cephalexin at their respective MRLs.

Table 6. Detection capability (CCβ) of the Betastar Combo, SNAP and Twinsensor[BT] tests for beta-lactams and tetracyclines in sheep milk

Antibiotics	MRL[1] (µg/kg)	CCβ[2] (µg/kg)		
		Betastar Combo	SNAP[3]	Twinsensor[BT]
Beta-lactams				
Amoxicillin	4	4	4	4
Ampicillin	4	3	3	≤ 2
Benzylpenicillin	4	≤ 2	≤ 2	3
Cloxacillin	30	≤ 15	20	≤ 15
Dicloxacillin	30	≤ 15	≤ 15	≤ 15
Nafcillin	30	≤ 15	30	> 30
Oxacillin	30	≤ 15	30	≤ 15
Cefacetrile	125	≤ 63	≤ 63	≤ 63
Cefalonium	20	≤ 10	20	≤ 10
Cefapirin	60[4]	≤ 30	≤ 30	≤ 30
Cefazolin	50	50	≤ 25	≤ 25
Desacetylcefapirin	*	≤ 30	≤ 30	≤ 30
Cefoperazone	50	≤ 25	≤ 25	50
Cefquinome	20	≤ 10	≤ 10	20
Ceftiofur	100[5]	> 100	≤ 50	≤ 50
Desfuroylceftiofur	*	> 100	≤ 50	≤ 50
Cephalexin	100	> 100	≤ 50	> 100
Tetracyclines				
Chlortetracycline	100[6]	100	100	100
4-epichlortetracycline	*	> 100	> 100	> 100
Oxytetracycline	100[6]	100	≤ 50	75
4-epioxytetracycline	*	> 100	> 100	> 100
Tetracycline	100[6]	100	75	100
4-epitetracycline	*	> 100	> 100	> 100

[1]MRL: Maximum Residue Limit established by EC Regulation Nº 37/2010; [2] CCβ (µg/kg): antibiotic concentration that produces at least 95 % positive results; [3]SNAP tests: SNAP Betalactam test and SNAP Tetracycline test; [4]sum of cefapirin and desacetylcefapirin; [5]sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur; [6]sum of parent drug and its 4-epimer; *marker residue. MRL not established

4. Conclusions

Despite the differences in terms of chemical composition and hygienic quality of milk from sheep to cows which could have affected the performance of these screening tests, the results obtained indicate that all the receptor-binding tests assessed are suitable for use in raw sheep milk. Thus, the Betastar Combo, SNAP Betalactam, SNAP Tetracycline and Twinsensor[BT] tests, presented high specificity values (≥ 99 %) for the

detection of beta-lactams and tetracyclines in bulk sheep milk. Also, individual sheep milk samples were analysed successfully with the SNAP and Betastar Combo tests. The Twinsensor[BT] test not should be used with this type of milk, especially at the end of the lactation period because of the high probability of finding false-positive results. Azidiol as a preservative had no influence on the test performance and neither did the presence of antibiotics other than beta-lactams and tetracyclines in milk. In addition, the CCβ of the tests investigated was at or below MRL for most antibiotics considered. In general, no significant differences were found between visual and instrumental interpretation of the test results, which allows the use of these tests with or without a specific reader. In conclusion, the Betastar Combo test (Neogen Corporation), the SNAP Betalactam test (IDEXX Laboratories), the SNAP Tetracycline test (IDEXX Laboratories) and the Twinsensor[BT] test (Unisensor), are suitable for routinary screening of antibiotics in bulk milk from ovine livestock.

5. Acknowledgments

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Validation of receptor-binding assays to detect antibiotics in goat's milk

Beltrán M.C.[a], Borràs M.[a], Nagel O.[b], Althaus R.L.[b], Molina M.P.[a]

[a]Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera, s/n, 46071 Valencia, Spain.

[b]Cátedra de Biofísica, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P.L. Kreder 2805, 3080 Esperanza, Argentina.

Abstract

The suitability of different receptor-binding assays to detect antibiotics in raw goat milk was investigated. Detection capability (CCβ) of most beta-lactams and tetracyclines assessed applying the Betastar Combo, the SNAP Betalactam, the SNAP Tetracycline and the Twinsensor[BT] tests was at or below maximum residue limits (MRLs) established by European legislation. Regarding test specificity, cross-reactions with antibiotics other than beta-lactams and tetracyclines were not found, and no false-positive results were obtained for the Betastar Combo and the SNAP tests when goat bulk milk samples were analyzed. For the Twinsensor[BT] test, the false-positive rate was 1 %. The performance of the Betastar Combo and the SNAP tests was practically unaffected by the milk quality parameters using individual goat milk collected along the entire lactation period (false-positive rate ≤ 5 %). However, a larger number of positive results was obtained by the Twinsensor[BT] test in this type of milk samples (> 10 %), especially in the last weeks of lactation. Interferences related to the use of the preservative azidiol were not observed in any case. Neither were any significant differences found in relation to the interpretation method (visual *vs* instrumental) applied. In general, the response of the Betastar Combo, SNAP and Twinsensor[BT] tests was optimal for the analysis of caprine bulk milk, thus they may be used in a satisfactory manner to monitor milk for the presence of beta-lactam and tetracycline residues in quality control programs.

Chapter 5

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

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

Beltrán M.C.[a], Althaus R.L.[b], Molina A.[c], Berruga M.I.[c], Molina M.P.[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.

[c]Departamento de Ciencia y Tecnología Agroforestal y Genética, Universidad de Castilla-La Mancha, 02071 Albacete, Spain.

Abstract

The production systems of the Spanish dairy sheep and goats sectors have been intensified in recent years, and in this context the use of antimicrobials to treat and prevent infectious diseases is a widespread practice that can result in the contamination of the milk supply. Mastitis is undoubtedly the infectious disease most frequently treated with antibiotics in dairy sheep and goat livestock, using primarily beta-lactam drugs, while macrolides constitute the second most important group of antimicrobials applied. Therefore, substances belonging to these groups 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. For sheep and goat's milk, Spanish regulation establishes the control of the presence of antibiotic residues using methods that detect, at least, beta-lactam drugs. Microbial inhibitor tests using *Geobacillus stearothermophilus* var. *calidolactis* and specific receptor-binding assays are widely used for screening antibiotics in milk. Taking into account the frequency of use of antibiotics commonly employed by veterinarians to treat and prevent mastitis in lactation, detection rates of screening tests routinely applied in Spain have been evaluated in order to propose an analytical strategy based on the use of these methods to detect residues of antibiotics in a simple and economic manner. 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 in order to carry out a more efficient screening and ensure the safety of milk and dairy products from sheep and goats.

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1. 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 and Piredda, 2007).

The quality of sheep and goat's milk has increased significantly, not only in terms of their fat- and protein contents, but also with regard to hygienic quality. In this sense, the establishment of Community legislation concerning the hygiene of foodstuffs of animal origin intended for human consumption (Regulation EC N° 853/2004 and amendments) 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 indicated 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 and 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 (Regulation UE N° 37/2010) is mandatory in countries of the European Union (Regulation EC N° 853/2004 and amendments). 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 with the fermentation processes required for the manufacture of certain 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). 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 (Real Decreto 752/2011) 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 must 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 sheep and goats, 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 in Spain.

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.

2. Material and methods

2.1. 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, Hiller∅d, Denmark), somatic cell count (Fossomatic 5000, Foss), total bacterial count (Bactoscan FC, Foss), and pH value (pHmeter, Crison, Barcelona, Spain) to check their suitability to be used as "negative milk" according to the IDF recommendations (ISO/IDF, 2002 and 2003).

2.2. 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 Produktions-und 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), 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 test results were classified as positive or negative both, visually by three trained

technicians, and instrumentally by specific devices provided by manufacturers, except for the Delvotest MCS Accelerator which only instrumental reading was performed.

2.3. Antimicrobials and spiked milk samples

Antimicrobials most employed by veterinarians to treat and prevent mastitis in dairy sheep and goats were selected for this study. In agreement to Berruga et al. (2008) who surveyed veterinarians for information on antimicrobial treatments most commonly applied in Spain to treat and prevent infectious diseases in dairy sheep and goats, 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), sulfadimethoxine (S7385), 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, 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, 2002 and 2003), 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.

2.4. Calculation of the total detection rate for screening tests

Taking into account the frequency of use of each "a" antimicrobial substance (F_a), calculated from data provided by Berruga et al. (2008) and the "t" test sensitivity

for each antibiotic at MRL equivalent concentration (SMRL$_{t,a}$), the detection rates of each screening test ($DR_{t,a}$ (%)= $F_a \cdot$SMRL$_{t,a}$) were calculated.

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

$$TDR_t = \sum_{a=1}^{a=n} DR_{t,a}$$

(Eq. I)

2.5. 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:

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

(Eq. 2)

where:

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

2.6. 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).

3. Results and discussion

3.1. Detection rates of antimicrobials in sheep milk

Table 1 summarizes the frequency of use of antibiotics most commonly applied in Spain to treat mastitis in dairy sheep, the sensitivities of the microbial screening tests and the detection rates calculated for each antibiotic substance depending on its

frequency of use ($DR_{M,A}$). Information related to rapid receptor tests is presented in Table 2.

Table 1. Detection rate of antibiotics reached by microbial screening tests in sheep milk

Antimicrobials	F[1]	SMRL[2] (%)				DR[3] (%)			
		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	29.10	29.10	29.10	29.10
Amoxicillin	15.00	100	100	100	100	15.00	15.00	15.00	15.00
Cloxacillin	14.20	100	100	100	100	14.20	14.20	14.20	14.20
Ceftiofur	3.10	45	100	100	100	1.40	3.10	3.10	3.10
Ampicillin	2.40	100	100	100	95	2.40	2.40	2.40	2.28
Cephalexin	2.40	47	100	100	100	1.13	2.40	2.40	2.40
Cefquinome	1.60	0	0	0	0	0.00	0.00	0.00	0.00
Cefoperazone	1.60	58	77	32	15	0.93	1.23	0.51	0.24
Cefazolin	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Cefalonium	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Cefapirin	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Cefacetrile	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Macrolides									
Erytromycin	6.70	96	17	8	5	6.43	1.14	0.54	0.34
Tylosin	6.40	100	100	100	100	6.40	6.40	6.40	6.40
Spiramicin	3.90	0	0	0	0	0.00	0.00	0.00	0.00
Lincomicin	1.80	82	23	68	5	1.48	0.41	1.22	0.09
Tetraciclinas									
Oxitetracycline	2.30	0	5	25	32	0.00	0.12	0.58	0.74
Tetracycline	0.50	0	3	0	0	0.00	0.00	0.00	0.00
Quinolones									
Enrofloxacin	3.80	2	0	0	0	0.08	0.00	0.00	0.00
Marbofloxacin	1.20	0	0	0	8	0.00	0.00	0.00	0.10
Aminoglycosides									
Streptomicin	0.30	16	0	0	8	0.05	0.00	0.00	0.02
Gentamicin	0.30	100	17	15	5	0.30	0.05	0.05	0.02
Neomycin	0.10	100	100	100	70	0.10	0.10	0.10	0.07
Sulphonamides									
Sulfametoxine	0.10	80	33	55	93	0.08	0.03	0.06	0.09
Sulfadiazine	0.10	60	100	100	100	0.06	0.10	0.10	0.10

[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; [3]DR: Detection rate for each antimicrobial substance considered

As shown in Table 1, although microbial screening tests have the same analytical basis they display different sensitivities in the detection of antibiotic substances. Thus, for example, molecules such as benzylpenicillin, cefalonium or tylosin are detected by all microbial inhibitor tests (sensitivity of 100 %), 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 milk.

In general, microbial screening tests present a high sensitivity for the detection of beta-lactam antibiotics (Beltrán et al., 2014). 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 by any of the microbial tests considered.

Table 2. Detection rate of antibiotics reached by receptor-binding assays in sheep milk

Antimicrobials	F[1]	SMRL[2] (%)				DR[3] (%)			
		Charm MRL	Betastar Combo	SNAP[4]	Twin-sensor	Charm MRL	Betastar Combo	SNAP[4]	Twin-sensor
Beta-lactams									
Benzylpenicillin	29.10	100	100	100	100	29.10	29.10	29.10	29.10
Amoxicillin	15.00	95	98	98	97	14.25	14.70	14.70	14.55
Cloxacillin	14.20	18	100	100	100	2.55	14.20	14.20	14.20
Ceftiofur	3.10	100	92	100	100	3.10	2.85	3.10	3.10
Ampicilin	2.40	97	100	100	100	2.33	2.40	2.40	2.40
Cefalexin	2.40	100	0	100	0	2.40	0.00	2.40	0.00
Cefquinome	1.60	100	100	100	100	1.60	1.60	1.60	1.60
Cefoperazone	1.60	100	100	100	100	1.60	1.60	1.60	1.60
Cefazolin	0.80	100	95	100	100	0.80	0.78	0.80	0.80
Cefalonium	0.80	100	100	95	100	0.80	0.80	0.76	0.80
Cephapirin	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Cefacetrile	0.80	100	100	100	100	0.80	0.80	0.80	0.80
Tetracyclines									
Oxitetracycline	2.30	100	97	100	100	2.30	2.23	2.30	2.30
Tetracycline	0.50	100	97	100	97	0.50	0.49	0.50	0.49

[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, [3]DR: Detection rate for each antimicrobial substance considered; [4]SNAP: SNAP tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously

Similar results were reached with the receptor-binding assays studied. Of all the beta-lactams and tetracyclines used in lactating dairy sheep, 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 method.

Using Equation 1 presented in the Materials and Methods section, the total detection rate for screening tests (TDR_t) was calculated and is summarized in Table 3.

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

Microbial tests	TDR (%)	Receptor-binding assays	TDR (%)
BRT MRL	82.1	Charm MRL BLTET	62.8
Delvotest MCS SP-NT	78.9	Betastar Combo	72.2
Delvotest MCS DA	78.8	SNAP[1]	74.9
Eclipse 100	77.2	Twinsensor[BT]	72.4

[1]SNAP: SNAP 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 (82.1 %) 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 (62.8 - 74.9 %) with this test type when all antimicrobials are considered.

When comparing the total detection rates achieved with microbial and rapid receptor screening tests, respectively, through the Mann-Wilcoxon contrast test, significant differences between the two types of assays were found (W= 16.0 and 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.

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

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

	Betastar	Charm	SNAP[1]	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
Accelerator								78.9
Eclipse								

[1]SNAP: SNAP tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously

As shown in Table 4, the simultaneous use of two microbiological screening methods 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 obtained with the use of a single screening test belonging to this group (W= 20.5 and 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 and 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 and p= 0.0005) allowing, therefore, a more efficient control of antibiotic residues in sheep milk.

3.2. Detection of antimicrobials in goat's milk

Tables 5 and 6 summarize the frequencies of use, sensitivities at MRLs of microbial inhibitor tests and receptor-binding assays, respectively, as well as their

detection rates for the different antibiotics usually applied in the treatment of mastitis in goats.

As explained previously for sheep milk, microbial screening tests display a high sensitivity for the detection of beta-lactam antibiotics (Table 5), and can also detect some of the remaining substances considered at legally established safety levels. On the other hand, beta-lactam and tetracycline antibiotics are widely detected by rapid receptor tests reaching total detection rates higher than those indicated previously for sheep milk (Table 6).

Table 5. Detection rate of antibiotics reached by microbial screening tests in goat's milk

Antimicrobials	F[1]	SMRL[2] (%)				DR[3] (%)			
		BRT MRL	Delvo SP-NT	Delvo DA	Eclipse 100	BRT MRL	Delvo SP-NT	Delvo DA	Eclipse 100
Beta-lactams									
Benzylpenicillin	30.00	100	100	100	100	30.00	30.00	30.00	30.00
Amoxicillin	20.00	100	100	97	95	20.00	20.00	19.40	19.00
Cloxacillin	10.00	100	100	100	97	10.00	10.00	10.00	9.70
Ceftiofur	3.60	47	80	73	92	1.69	2.88	2.63	3.31
Ampicillin	2.10	100	100	100	100	2.10	2.10	2.10	2.10
Cephalexin	1.40	35	100	100	100	0.49	1.40	1.40	1.40
Cefquinome	0.70	33	28	12	3	0.23	0.20	0.08	0.02
Cefoperazone	0.70	48	83	58	28	0.34	0.58	0.41	0.20
Cefazolin	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefalonium	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefapirin	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefacetrile	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Macrolides									
Erytromycin	5.10	100	45	10	17	5.10	2.30	0.51	0.87
Tylosin	3.20	100	100	100	95	3.20	3.20	3.20	3.04
Spiramicin	2.20	0	0	0	0	0.00	0.00	0.00	0.00
Lincomicin	1.20	55	40	80	3	0.66	0.48	0.96	0.04
Tetraciclinas									
Oxitetracycline	8.20	38	20	30	47	3.12	1.64	2.46	3.85
Tetracycline	1.60	0	5	5	5	0.00	0.08	0.08	0.08
Quinolones									
Enrofloxacin	3.60	0	0	0	0	0.00	0.00	0.00	0.00
Marbofloxacin	0.70	0	0	0	0	0.00	0.00	0.00	0.00
Aminoglycosides									
Gentamicin	1.20	97	13	5	5	1.16	0.16	0.06	0.06
Neomycin	1.20	100	100	100	65	1.20	1.20	1.20	0.78
Streptomicin	0.40	25	5	5	25	0.10	0.02	0.02	0.10

[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, [3]DR: Detection rate for each antimicrobial substance considered

The total detection rates for screening tests using goat's milk are presented in Table 7. The BRT and the SNAP tests displayed the highest percentages of drugs detected for the two test types considered.

Table 6. Detection rate of antibiotics reached by receptor-binding assays in goat's milk

Antimicrobials	F[1]	SMRL[2] (%)				DR[3] (%)			
		Charm MRL	Betastar Combo	SNAP[4]	Twin-sensor	Charm MRL	Betastar Combo	SNAP[4]	Twin-sensor
Beta-lactams									
Benzylpenicillin	30.00	100	100	100	100	30.00	30.00	30.00	30.00
Amoxicillin	20.00	95	100	100	100	19.00	20.00	20.00	20.00
Cloxacillin	10.00	15	100	100	100	1.50	10.00	10.00	10.00
Ceftiofur	3.60	100	90	100	100	3.60	3.24	3.60	3.60
Ampicilin	2.10	97	100	100	100	2.04	2.10	2.10	2.10
Cefalexin	1.40	100	5	100	0	1.40	0.07	1.40	0.00
Cefquinome	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefoperazone	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefazolin	0.70	100	97	100	100	0.70	0.68	0.70	0.70
Cefalonium	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cephapirin	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Cefacetrile	0.70	100	100	100	100	0.70	0.70	0.70	0.70
Tetracyclines									
Oxitetracycline	8.20	100	100	100	100	8.20	8.20	8.20	8.20
Tetracycline	1.60	95	100	100	100	1.52	1.60	1.60	1.60

[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, [3]DR: Detection rate for each antimicrobial substance considered, [4]SNAP: SNAP tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously

The total detection range of microbial inhibitor tests (77.4 - 82.3 %) and that of rapid receptor tests (71.6 - 82.4 %) are similar, although receptor-binding assays detect only beta-lactam and tetracycline antibiotics, while microbial methods can also detect other drugs such as neomycin, gentamicin, or sulphonamides. Therefore, from a food safety point of view, the application of one test only seems insufficient as an appreciable percentage of antibiotic residues remain undetected and could thus reach the consumer.

The application of the Mann-Wilcoxon test to compare the total detection ranges obtained in both groups of the screening tests did not show significant differences (W= 0.3806 and 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.

Table 7. Total detection rate (TDR) of antibiotics reached by screening tests in goat's milk

Microbial tests	TDR (%)	Rapid receptor tests	TDR (%)
BRT MRL	82.3	Charm MRL BLTET	71.6
Delvotest MCS SP-NT	79.1	Betastar Combo	79.5
Delvotest MCS DA	77.4	SNAP	82.4
Eclipse 100	77.4	Twinsensor[BT]	79.8

[1]SNAP: SNAP tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously

The simultaneous use of two microbial screening tests (Table 8) does not lead to an improvement of the total detection ranges in comparison to the use of a single test (W = 21.0 and p = 0.069) leaving a percentage of undetected residues ranging from 14.4 to 20.1 %.

Table 8. Detection rates of antibiotics reached with the simultaneous use of two screening tests in goat's milk

	Betastar	Charm	SNAP[1]	Twinsensor	BRT	Delvotest	Accelerator	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
Accelerator								79.9
Eclipse								

[1]SNAP: SNAP tests are considered here as a combined test able to detect beta-lactams and tetracyclines simultaneously

Similarly, when applying the Mann-Wilcoxon test to compare the total detection rates obtained by the application of a single rapid receptor test (Table 4) with those calculated when two receptor-binding assays were used simultaneously (Table 8), significant differences were not found (W= 21.0 and 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 and p= 0.001) or only a microbial method (77.4 and 82.3 %, W = 96.0 and 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.

It should be kept in mind that Spanish legislation (Real Decreto 752/2011) 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 by most operators in the sector for screening antibiotics in raw milk from sheep and goats, is rather appropriate as it 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 (Real Decreto 1728/2007) 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 carried out on an obligatory basis in one out of five tankers, assuring 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 related 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 increasing the spectrum of detection and minimize the risks derivate of the presence of these residues in milk.

4. Conclusions

The simultaneous use of two screening tests with a different analytical basis allows achieving a broader coverage of the antimicrobial substances 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

5. Acknowledgements

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Chapter 6

General discussion

1. General aspects

Antibiotic therapy is an effective and useful method of treating bacterial infections in dairy livestock. Used appropriately, antibiotics help to maintain animal health and well-being as well as milk production and reduce the risk of pathogenic bacteria in milk. The improper use of drugs in the treatment of lactating dairy animals can result in milk containing drug residues above target Maximum Residue Limits (MRLs), making milk unsuitable for human consumption and for dairy product manufacturing.

The presence of antibiotic residues in milk has negative repercussions on the technological properties as they can totally or partially inhibit fermentation procedures required when making cheese and yoghurt (Packham et al., 2001). Sheep and goats milk are basically destined for the elaboration of fermented products, and antibiotics in milk can thus affect the production process; also, as residues of variable amounts may remain in the final products, consumer safety might be compromised (Oliver et al., 2011).

For these reasons, integrated dairy chain management must employ an efficient detection system to verify the safety of milk through the application of relevant detection methods at key steps of the chain to rule out the presence of these substances above legally established limits.

Qualitative antibiotic screening tests are most frequently used along the dairy chain from farm to dairy processing. Results are obtained as positive or negative and the most widely used tests can be classified in two categories: microbial inhibitor tests and receptor-binding assays. These tests have been developed for the use in cow milk and, therefore, their validation in sheep and goat's milk according to guidelines for analytical method validation issued by International regulatory authorities is necessary.

This thesis focuses on the tools currently available on the market to establish an appropriate system for the detection of antibiotic residues in sheep and goat's milk. It aims to provide guidance on how to screen antibiotic residues in small dairy ruminants within the dairy chain to ensure food safety and, ultimately, to deliver safe dairy products to consumers.

2. Microbial inhibitor tests

Microbial inhibitor tests are routinely applied in quality control laboratories to check the presence of antibiotic residues in raw milk as they are relatively inexpensive, user-friendly, and able to detect a great variety of antimicrobials in a large number of milk samples. Most current microbial screening tests were initially developed to detect beta-lactams in cow milk and are based on the inhibition of *Geobacillus stearotermophilus* var. *calidolactis* being highly sensitive to these substances. However, they are not suitable for the detection of most drugs belonging to other antimicrobial groups.

To improve test sensitivity, in particular towards non-beta-lactam drugs, various authors proposed some modifications such as the addition of chelating agents or antifolates, such as trimethoprim, into the culture medium, to enhance the detection of tetracyclines and sulphonamides in milk, respectively (Adriany et al.,1995; Langeveld et al., 2005). In recent years, manufacturers have improved some performance characteristics of microbiological methods, especially the time required for the analyses, and sensitivity to different substances, and new versions of these tests are now available (ISO/IDF, 2010).

In fact, the first versions of the microbial screening tests showed good detection limits for most beta-lactam antibiotics in sheep milk but did not detect tetracycline and quinolone residues. Thus, in a study conducted by Molina et al. (2003a) the BRT test (AiM) presented very high detection limits for antimicrobials such as erythromycin (630 µg/Kg), tylosin (120 µg/Kg), gentamicin (1,200 µg/Kg), neomycin (3,700 µg/Kg), sulfadiazine (5,400 µg/Kg), sulfametoxazol (3,200 µg/Kg) and sulfaquinoxalina (6,200 µg/Kg). However, the current version of this test, BRT MRL test, presents detection capabilities (CCβs) at or below regulatory limits (MRLs) for gentamicin, neomycin, erythromycin, tylosin and sulfadimethoxine (Beltrán et al., 2014a).

The Delvotest SP version (DSM Food Specialties) evaluated by Althaus et al. (2003a) in sheep milk, presented higher detection limits for tylosin (100 µg/Kg) and neomycin (6,200 µg/kg) while its current versions, Delvotest MCS SP-NT and Delvotest MCS Accelerator (DSM Food Specialties), are able to detect neomycin (CCβ ≤ 750 µg/Kg), tylosin (CCβ ≤ 25 µg/Kg), sulfadiazine (CCβ= 75 µg/Kg) and sulfadimethoxine (CCβ ≤ 50 µg/Kg) at safety levels (Beltrán et al., 2014a).

Montero et al. (2005) employing the Eclipse 100ov test (Zeulab) found higher detection limits for neomycin (9,100 µg/Kg) and tylosin (230 µg/Kg), respectively. However, the latest Eclipse 100 test (Zeulab) also presents a CCβ at MRL for tylosin

(50 µg/Kg) and increased sensitivity (70 %) for the detection of neomycin at MRL equivalent to the antibiotic concentration (1,500 µg/Kg) in sheep milk samples.

Studies conducted by Sierra et al (2009a,b) in goat's milk also indicate detection capabilities at or below maximum residue limits (MRLs) for some beta-lactam drugs and lower ability to detect tetracyclines and quinolones. Thus, the BRT (AiM), Delvotest SP and Eclipse 100 tests showed higher detection limits for gentamicin (353 µg/Kg, 353 µg/Kg, and 555 µg/Kg, respectively), erythromycin (174 µg/Kg, 174 µg/Kg and 437 µg/Kg, respectively), lincomycin (264 µg/Kg, 264 µg/Kg and 931 µg/Kg, respectively), sulfamethazine (555 µg/Kg, 183 µg/Kg and 1,269 µg/Kg, respectively) with respect their respective MRLs.

Based on the results obtained herein, it can be concluded that microbial screening tests such as the BRT MRL, the Delvotest MCS SP-NT, the Delvotest MCS Accelerator and the Eclipse 100 are suitable for the detection of beta-lactam antibiotics, sulphonamides, and some macrolides (erythromycin and tylosin) in sheep and goat's milk. However, *Geobacillus stearothermophilus* does not display adequate sensitivity to detect residues of tetracyclines and quinolones at levels of their MRLs.

To widen the detection spectrum of antibiotic residues in milk, some authors proposed the combinations of different test bacteria, each in an optimal medium, that act in a complementary manner to detect a large range of veterinary drugs up to the MRL levels (Nows et al., 1999; Gaudin et al., 2004; Althaus et al., 2009). However, these screening methods are laborious and require qualified staff for the preparation of the petri dishes containing the inoculated culture medium and the interpretation of the results from the measurement of the inhibition halos generated as a result of the presence of antibiotic residues in milk after, usually, long incubation periods (16-24 h).

In this sense, Nagel (2013a) proposes the use of chemometric techniques that employ multiple logistic regression models and the desirability function to design and optimise a microbiological bioassay in microtiter plates with a dichotomous response using complementary bacteria such as the *Geobacillus stearothermophilus* and *Bacillus subtilis*. Thus, *B. subtilis* acts as a complementary bacterium of *G. stearothermophilus* as it presents a greater sensitivity for the detection of ciprofloxacin, enrofloxacin, marbofloxacin, erythromycin, and spiramycin.

In this thesis the detection system based on the use of two microplates containing *Geobacillus stearothermophilus* and *Bacillus subtilis*, respectively, in milk from sheep has been assessed (Nagel et al., 2012), obtaining good detection limits for residues of quinolones (160 µg/l of ciprofloxacin, 230 µg/l of enrofloxacin and 280 µg/l

of marbofloxacin) and macrolides (60 μg/l of erythromycin and 380 μg/l of spiramycin) when compared with the high detection limits of commercial screening tests using *G. stearothermophilus*.

Therefore, the combination of different microtiter plates (Althaus et al., 2014) could be an alternative of interest to improve the detection rate of potential drug residues in milk, in a simple, relatively fast (6 h) and economical manner.

Recent studies have shown a trend towards the development of more rapid microbiological methods using thermophilic bacteria to reduce the total incubation period. Nagel et al. (2013b, 2014) using bioassays employing *Geobacillus thermoleovorans* and *Geobacillus thermocatenulatus*, respectively, reached suitable results (CCβ ≤ MRLs) for the detection of beta-lactams in milk in less than 2.5 hours.

Another important aspect in the evaluation of the performance of microbial screening tests is the specificity that considers the probability that the test will be negative among samples which do not contain residues of the target analyte. Microbial screening tests are not specific for antibiotics and might react with other types of inhibitors present in milk (Carlsson et al., 1989; Andrew et al., 1997). Previous studies carried out in sheep milk have laid bare apparent interferences related to a high fat content, and elevated somatic cell count or the presence of natural inhibitors in milk (Althaus et al., 2003b).

Results obtained in this thesis suggest that microbial screening tests showed an elevated percentage of non-compliant results (4.8-10 %) when antimicrobial-free sheep milk samples were analysed (Beltrán et al., 2014a) and interferences were related to an elevated somatic cell count (SCC) in all cases. These results are in agreement with those obtained by others authors (Cullor et al., 1992) who also observed a significant effect of SCC on the frequency of positive results for different screening tests using individual milk samples from cows. However, the test responses were unaffected by goat milk properties, including SCC, when antimicrobial-free milk samples were analysed; and the false-positive outcomes recorded were below 5 % in all cases.

It should be noted that in quality control programmes raw bulk milk samples are usually analysed, not individual milk samples, presenting a minor range of variation in all quality parameters, and very low percentages of non-compliant results were obtained when microbial screening test were used (Comunian et al., 2010). However, farmers need sometimes to test individual milk samples from a treated animal to ensure that no drug residues will contaminate their production. This is particularly recommended when in doubt concerning the observation of good practices in treated

animals (application of withdrawal period, identification of a treated animal, non-cured animal, etc.). In this sense, knowing the response of the microbial screening tests when using individual samples is essential. The results of the thesis indicate that microbial screening tests are not suitable to check drug residues in individual milk samples from sheep as they can lead to false-positive results especially for samples containing a higher SCC.

3. Rapid screening tests

To reduce the test time response bioanalytical methodologies have been proposed, employing specific protein receptors to beta-lactams located in cells membranes (Charm, 1980 a,b, Degelaen et al., 2003), and including lateral flow chromatography in reactive dipsticks (Markovsky, 2001, 2006).

At present, rapid screening tests based on the use of specific receptors are widely used, especially on farms and dairies where a fast response (< 10 minutes) is required. Their detection spectrum is normally limited to one group of antibiotics, generally beta-lactams or tetracyclines, although in recent years test versions have appeared able to detect the two antibiotic groups simultaneously. Receptor-binding assays have been validated for the use in raw bulk milk from cows (Perme et al., 2010; Reybroeck et al., 2010; Salter et al., 2011), but information on their performance characteristics in sheep and goat's milk is rather limited.

Some of the rapid receptor tests (the Betastar Combo, Charm MRL BLTET, SNAP and Twinsensor[BT]) have been evaluated for their use in sheep and goat's milk (Beltrán et al. 2013; Beltrán et al., 2014b,c), presenting detection capabilities equal or lower to the MRLs for most beta-lactams and tetracyclines. Moreover, although receptor-binding test results can be classified using specific readers for a more objective interpretation, significant differences were not found between the visual and instrumental reading of the results.

The specificity of rapid receptor tests was optimal in all cases. No cross-reactions were found when drugs belonging to antimicrobial groups other than beta-lactams or tetracyclines were present in milk. The false-positive rate was lower even when individual sheep and goat's milk samples were analysed in most cases. Only the Twinsensor[BT] test presented a larger number of positive results (> 10 %) when antibiotic-free milk samples from individual sheep and goats were analysed (Beltrán et al., 2014b,c), especially in the last weeks of lactation.

For sheep and goat's milk quality control programmes, Spanish legislation (Real Decreto 752/2011) establishes the use of azidiol as a preservative in milk sampling,

stipulating its composition and dosage in samples from sheep and goats. The effect of azidiol on the test response has been evaluated in microbial screening tests, finding an increased occurrence of false positive results in samples spiked with the preservative (Molina et al., 2003b), although these interferences can be minimized through the extension of the recommended incubation time (Molina et al., 1999). However, information on the effect of azidiol on the receptor-binding test response is practically non-existent.

The results herein thesis indicate that azidiol had no effect on the performance of the receptor-binding tests assessed in both, sheep and goat's milk (Beltrán et al., 2013; Beltrán et al., 2014a,b). No change in negative or positive results was observed, neither visually nor instrumentally, when antibiotic-free milk and samples spiked with antibiotics, with and without azidiol were analysed. However, using the SNAP Betalactam test, a delay in the appearance of the coloured spots was observed when milk with azidiol were analysed, making a prolonged incubation period necessary. In addition, the coloured spots had a lower intensity, making the visibility of the control spot more difficult, which may in turn complicate the visual interpretation of the results.

At present, more sensitive, faster, multiple, high–throughput and cost-effective methods for the simultaneous determination of residual veterinary drugs in milk are being developed. In fact, in 2013, versions of lateral flow chromatographic receptor-binding tests such as the BetaXpress Milk MRL (Unisensor, Belgium) and the Charm MRL 1 (Charm Sciences Inc., USA) were launched, making the detection of beta-lactams much faster, i.e. requiring only three, respectively, one minute only, and having been validated for the routine screening of milk by Reybroeck and Ooghe (2014a,b).

Other rapid receptor tests for the simultaneous detection of two or more groups of antimicrobials have been developed, some of them not yet available in Spain, such as SNAP duo (IDEXX Laboratories), Twinexpress[BT] (Unisensor), Trisensor (Unisensor), 4sensor BSCT (Unisensor), 4sensor BTSQ (Unisensor) and 4sensor BTGQ (Unisensor). Some of these tests are currently in the process of validation according to Commission Decision 2002/657/EC and the Guidelines for the validation of screening methods proposed by Community Reference Laboratories for residues (CRLs, 2010) at ILVO (Institute for Agricultural and Fisheries Research, Belgium) or other accredited laboratories.

The technological development in recent years has not only focused on receptor-binding assays but also on other analytical methods such as ELISA test kits (Tecna, Italy; r-Biopharm, Germany; Randox Food Diagnostics, United Kingdom),

biochip array technology (Evidence Investigator, Randox Food Diagnostics) and flow cytometry immunoassays (BeadYplex, Unisensor) covering beta-lactams, tetracyclines, sulphonamides, macrolides, aminoglycosides, lincosamides, polymyxins and quinolones.

As a consequence of the great analytical advances made, more tools to screen antibiotic residues in an easier manner are available at an acceptable cost.

4. Analytical strategy

The sampling and testing of antibiotics in milk should be carried out by producers, collectors or processors of milk in the context of a national or regional control scheme. The European Union is rather vague about how and when the testing of antimicrobials must be performed and establishes that each member state ought to set up its own system of control of the quality and traceability of milk (Regulation EC Nº 178/2002). In this sense, Spanish legislation establishes the sampling and testing requirements at different stages for raw milk from cows (Real Decreto 1728/2007), respectively, raw milk from sheep and goats (Real Decreto 752/2011).

On farms, Spanish regulation for sheep and goat's milk has foreseen checks of farm bulk tanks when there is doubt or the certainty of the presence of antibiotics before loading the milk onto the tanker to prevent contamination. In general, on each farm, according to a sampling scheme, a representative sample of farm bulk milk is taken before the milk leaves the farm (ex-farm milk). Samples are stored at dairies and then collected by an independent laboratory. Depending on the operators, various methods are implemented according to the requirements.

In Spain, ex-farm milk testing by laboratories is enforced by regulation (minimum two samples per month) or by agreements between the dairy collector and the milk suppliers. The frequency for analysis may vary depending on dairy sector specifications, but the more frequently it is checked, the lower the risk of finding residues at later stages and ultimately in the final product.

At dairies, milk from tankers is systematically tested with a rapid test specific for beta-lactam antibiotics at each delivery prior to acceptance. If the result is negative, milk is unloaded, however, in the case of a positive result in the rapid screening test, milk from the tanker will be re-tested another method having the same detection patterns and a different analytical basis, i.e. microbial inhibitor test. If the result is positive, the milk is withdrawn, considered a category 2 animal by-product and has to be destroyed according to the most suitable method.

By considering the drugs used in a particular area and the risk of finding consecutive residues in milk, operators should choose appropriate screening tests depending on the specific circumstances, and the test kit should ideally operate at or near the MRL for the antibiotics used. The tests have specified limits of detections (CCβ), for targeted drugs covered by their scope.

In Spain, according to a survey conducted by Berruga et al. (2008) on the main causes of the presence of veterinary drug residues in milk from dairy sheep and goats livestock, mastitis is undoubtedly the infectious disease most frequently treated with antibiotics during the lactation period, using primarily beta-lactam drugs; macrolides being the second most important group of antimicrobials applied. Therefore, substances belonging to these groups are the most probable residues in raw milk from these species.

For sheep and goat's milk, Spanish regulation establishes the control of the presence of antibiotic residues using methods that detect, at least, beta-lactam drugs. Microbial inhibitor tests using *Geobacillus stearothermophilus* var *calidolactis* and receptor-binding assays specific for the detection of beta-lactams are most widely used for screening antibiotics in milk.

Taking into account the frequency of use of antibiotics commonly employed in Spain and the screening test sensitivity at MRLs, total detection rates have been calculated. 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 tests 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, respectively).

However, antibiotics such as enrofloxacin, marbofloxacin, spiramycin, and streptomycin also used to treat mastitis and other infectious diseases in sheep and goats could not be detected by the screening tests assessed. Therefore, the improvement of the analytical strategy through the periodical implementation of screening tests able to detect these substances at safety levels would be recommended.

In this sense, for example the implementation of a bioassay using *Bacillus subtilis* as bacteria-test would complement antibiotic coverage achieved by microbial

inhibitor tests using *G. stearothermophilus,* detecting substances such as erythromycin, enrofloxacin and spiramycin at concentrations more next to their respective MRLs. In addition, the periodic use of specific rapid methods for the detection of aminoglycosides and quinolones could be an alternative to increase the spectrum of detection.

Finally, we must consider that the presence of residues of antimicrobials in milk and the strategy used for their detection is dynamic and changes along time. The pharmaceutical industry constantly develops new formulae and products for the use in veterinary medicine. Also, manufacturers have developed new detection methods with an improved performance that have quickly been marketed in recent years. Also, many countries, concerned about food safety, implemented new legislative aspects. This implies that the analytical strategy should be revised periodically to adapt it to veterinary drugs employed by veterinarians in the area of the milk production and the new screening methodologies available.

The implementation of a proper analytical strategy could prevent the presence of antibiotic residues from reaching the food chain and, therefore, guarantee the safety of milk and dairy products.

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Chapter 7

Conclusions

Conclusions

Microbial inhibitor tests using *Geobacillus stearothermophilus* var. *calidolactis* such as the BRT MRL, the Delvotest MCS SP-NT, the Delvotest MCS Accelerator and the Eclipse 100 detect beta-lactam antibiotics and other non-beta-lactam drugs such as neomycin, tylosin, sulfadiazine and sulfadimethoxine in an efficient manner. However, they were less sensitive towards quinolones and tetracyclines at safety levels. When individual milk samples were analysed microbiological tests showed a higher occurrence of non-compliant results in sheep milk that were related in all cases to an elevated somatic cell count (SCC).

A microbiological system consisting of two bioassays using *Geobacillus stearothermophilus* var. *calidolactis* and *Bacillus subtilis,* respectively, presents a better sensitivity towards antibiotics such as quinolones, tetracyclines, lincomycin and spyramycin, with respect to the application of a commercial test using *G. stearothermophilus*. Therefore, this microbiological system proves to be a valuable tool to control ovine milk quality although it could be improved approaching some detection limits closer to established maximum residue limits (MRLs).

The Detection capability (CCβ) obtained for the receptor-binding tests, the Charm MRL BLTET, the Betastar Combo, the SNAP Betalactam, the SNAP Tetracycline and the Twinsensor[BT], was at or below MRL established by European regulation for most antibiotics considered indicating a high sensitivity towards beta-lactams and tetracyclines, except the CCβ for cloxacillin in the Charm MRL BLTET test and for cephalexin in the Betastar Combo and Twinsensor[BT] tests that were above their MRLs.

Regarding cross-reactions, interferences related to drugs other than beta-lactams and tetracyclines were not detected. Furthermore, the use of azidiol, as a preservative of milk, had no effect on the test response. Despite the differences in terms of chemical composition and hygienic quality of milk from sheep and goats *vs* cows, a higher specificity was obtained in all cases even when individual milk samples were analysed. Only the Twinsensor[BT] test presented non-compliant results using antibiotic-free milk samples from individual animals, especially in the last weeks of lactation.

Taking into account the performance of screening tests routinely applied in Spain and the frequency of use of antibiotics commonly employed by veterinarians in dairy sheep and goats, the total detection rates obtained were elevated using a microbial screening

test as well as a rapid receptor test although, the best antibiotic coverage was achieved with the simultaneous use of two screening tests with a different analytical basis (microbiological and receptor-binding tests). However, antibiotics such as enrofloxacin, marbofloxacin, spiramycin, and streptomycin are also employed by veterinarians in the antibiotic therapy of dairy sheep and goats and are not detected by the screening tests considered. To improve the analytical strategy, the periodical implementation of screening tests able to detect these substances at safety levels would be recommended to guarantee the quality of milk and dairy products from small ruminants.