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CONTROL AND PREVENTION OF ANTIBIOTIC RESIDUES AND CONTAMINANTS IN SHEPP AND GOAT'S MILK

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Highlights:

- Antibiotic residues in milk of small ruminants
- Causes and consequences of antibiotic residues in milk
- Control of antibiotic residues in milk
- Aflatoxins in milk of small ruminants
- Causes and consequences of aflatoxins in milk
- Prevention and control of aflatoxins in milk

Abstract:

The use of veterinary drugs to treat mastitis and other pathologies in dairy sheep and goats is a usual practice in current production systems. The risk of antibiotic residues in milk on farms is high if good farming practices are not applied, in this sense control measures must be implemented to prevent drugs residues from entering the food chain. But there are others compounds that may contaminate milk via the environment, water or animal feed, such as mycotoxins that are one of the most harmful contaminants given their negative effects on consumer health. This work presents the problems that arise when residues and contaminants are present in sheep and goat's milk. It also addresses the causes and the consequences of their presence, and the main measures of prevention and control required to guarantee milk that is safe for consumers and of high quality for the dairy industry.

Keywords: milk, sheep, goat, residues, antibiotics, contaminants, mycotoxins.

1. ANTIBIOTIC RESIDUES IN SHEEP AND GOAT'S MILK

The requirement to improve the quality of food items and to guarantee their safety led the European Union to enact a package of regulations to control food hygiene in 2004 (CE Regulation 852/2004), specifically for food of animal origin (CE Regulation 853/2004), including, among others, sheep and goat's milk. These regulations establish the hygiene-health parameters to evaluate in the raw milk of these animal species, and the aspects they safeguard include controlling residues of medicines for veterinary use and certain contaminants. The Maximum Residues Limits (MRL) for many of these substances are set out in the Annexe to Commission Regulation (EU) No. 37/2010.

1.1. Effect of the presence of antibiotic residues in milk

From a public health point of view, the presence of antimicrobial residues poses various problems, among which, the potential risks for consumers. Allergies to drugs, i.e. allergies to antibiotics like penicillin or amoxycillin represent over 40% of cases (Gamboa, 2009), which are the two most widely used antibiotics in treating infections of mammary glands in sheep and goats (Berruga et al., 2008a). The most frequent symptoms are cutaneous, and extreme cases include generalised anaphylactic reactions. Intake of foods that carry antimicrobial residues is also related to the development of microbial resistance (Philips et al., 2004).

The presence of such substances also affects the dairy industry as bacteria sensitive to antibiotics most widely used in many fermentation processes, result in spoiling the organoleptic properties of the final products, and coagulation or maturation of dairy products may fail. Studies into sheep milk yoghurt have observed that the levels of beta-lactam residues that come close to or below the MRL could delay coagulation by more than 40 minutes, cause variations in final compositions (Berruga et al., 2007a, 2008b; Novés et al., 2015), and could delay the pH in pressed cheeses from lowering by between 5-300 minutes (Berruga et al., 2007b). It is noteworthy that most heat treatments employed in the dairy industry have no strong impact on residues since they do, in general, not deactivate after heat treatment (Roca et al., 2010, Zorraquino et al., 2008).

An important aspect to consider is the possible economic impact of the presence of antibiotics in milk for the farmer as it may 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 (Category 2 Regulation CE 1069/2009).

Finally, residues from antibiotic treatments also have environmental implications as they can contaminate surface soil layers when eliminated through milk, urine and/or faeces, which might affect the microflora, microfauna and groundwater quality, having a serious impact on the natural environment (Kemper, 2008).

1.2. Main causes of antibiotic milk residues

Most studies concerning the cause of antimicrobial residues in milk have centred on cattle, although the reasons for such presence can be extrapolated to sheep and goat farms. Clinical mastitis and dry cow therapies are the cause of contaminated milk in most cases, however the crux of the problem is not the treatment itself, but lies in not correctly following good farming practices after applying treatment (Fabre et al, 1995). Negligence by workers is the main cause (30% of cases)according to Sánchez et al. (2001), and the inadequate use of antibiotics (29%) and the incorrect withdrawal (22%) are also relevant. The use of extra-label drugs treatments is also directly related as there are no specific withdrawal period for substances, except that established by law for this kind of treatments lasting at least 7 days (Directive 2001/82/EC).

The sector efforts made to reduce the number of samples contaminated by residues have resulted in quality comparable to that reached in cow milk. Nowadays, the percentage of positive samples in milk quality control laboratories is 0.1-0.001% (Gonzalo et al., 2012), and this figure is similar to that in neighbouring countries. Good farming practices and improvements in the quality control systems of milk of small ruminants have strongly influenced the progress made.

In order to adopt an effective control strategy, it is crucial to know the potential risk of the presence of antibiotic residues in sheep and goat's milk. To this end, the Spanish Ministry of Agriculture commissioned a national survey in 2008. The intention was to shed light on the pathologies that affect dairy sheep and goats, and the substance types used. This survey addressed veterinarians, revealing that mastitis was the main pathology. In fact, 72% of veterinarians in sheep and 77% in goats resorted to antibiotic therapy to treat mastitis during lactation (Berruga et al., 2008a). The reported substances mainly applied were beta-lactams (80%) and macrolides (36%) in sheep. The same pattern to treat mastitis was found in goats (80 and 27%, respectively). For dry therapy, antibiotics

(82% in sheep; 73% in goats) were reconfirmed, and beta-lactams and macrolides were also frequently used.

An outstanding finding in this study was, given the scarcity or inexistence of registered families of antibiotics for the use in small ruminants (macrolides or quinolones), the off-label use of antibiotics, evidencing the exceptional use of prescribed medicines (Directives 2001/82/EC and 2004/28/EC), and that suppression periods lasting at least 7 days are applied. Indeed, in the survey, 67% of veterinarians confirmed the use of prescribed drugs in sheep and 77% in goats, including quinolones, macrolides and cephalosporins. This use increases the risk of residues appearing, and several studies into sheep and goats have verified that this period is not always long enough to guarantee residue-free milk (Ferrini et al., 2010; Molina et al., 2003a).

3.1.Prevention and control of antibiotic residues in sheep and goat's milk

As the consequences of antibiotic residues being present in milk are serious, their absence must be guaranteed or if present they have to be at safe levels for consumers. Therefore, the aim is to prevent residues from reaching milk after applying treatments.

Stress should be placed on setting up self-control systems based on Good Farming Practices (CE Regulations 852/2004 and 853/2004). For sheep and goat's milk, the Spanish Ministry of Agriculture published guidelines in 2007 for Good Dairy Farming Practices (GDFP) concerning the production of raw milk from dairy animal species cover the main points of animal health, milk hygiene, nutrition, welfare, the environment and other related aspects. The GDFP areas most related to the presence of inhibitors in milk are "Animal Health," especially those aspects related to the use of chemicals and veterinary drugs and "Milking Hygiene," in everything related to the milking routine and the cleaning procedure of the surfaces that come into contact with milk. The main actions to be taken are identifying treated animals, separating milk from these animals, following a protocol to take action when mastitis appears, and respecting the suppression periods of medications during treatment.

In order to control antibiotic residues in milk, two analysis levels are considered (Decision 657/2002/CE); the first step in the residues control programme is in the screening phase in which a large numbers of samples originating from farms and the dairy industry are analysed and the second step is confirmation and quantification analyses. In these two steps different analytical methods are used (Figure 1).

In order to apply Community legislation with regard to the traceability of raw milk, in Spain Ministry of Agriculture issued Royal Decree 217/2004 created a software application to record the movements and quality controls of raw milk in Spain, i.e. the "Letra Q database" module (LEche, TRAzabilidad, Qualidad). Subsequently, Royal Decree 752/2011 was published establishing mandatory minimum controls to be performed by food-producing agencies, to harmonise the conditions required from laboratories for the analysis of raw milk from sheep and goats. Also minimum compulsory controls were established to detect antibiotic residues to be carried at the various production stages (farm and dairy), the actions to be taken in case of positive results and the characteristics of the detection methods.

For antibiotic detection, microbiological tests are the most widely used qualitative screening methods in control laboratories. These methods evidence inhibition of an indicator microorganism which, in most commercial tests, is *Geobacillus stearothermophilus* var. *calidolactis*. In Spain, the most frequently used commercial tests are BRT, Delvotest and Eclipse, which are well able to detect a large number of substances of the beta-lactam group and other antibiotics at their corresponding MRLs. Another screening method group is the receptor-based test, enabling the specific determination of a substance or antibiotic family above the level of interest. Some examples are the widely used commercial receptor-based tests in Spain: BetaStar, ROSA Charm, SNAP or Twinsensor.

1.4. Methods to control antibiotic residues in sheep and goat's milk

Most screening methods were originally developed for cow's milk, and have been introduced into routine sheep and goat's milk analyses, but differences in their composition have not been considered. Although the sensitivity of commercial microbiological screening methods has improved in recent years compared to that previously described (Althaus et al., 2001, 2003a, 2003b; Beltrán et al., 2015a; Montero et al., 2005; Sierra et al., 2009a, 2009b), they still have their limitations. Table 1 shows the results our group recently obtained (Beltrán et al., 2015a) with regard to the performance of microbiological inhibitor methods in sheep and goat's milk. The microbiological inhibitor methods are suitable for detecting beta-lactams, neomycin, tylosin, sulphadiazine and sulphadimethoxine in sheep and goat's milk, but show no optimum sensitivities to detect other antimicrobial groups, such as tetracyclines or quinolones at MRLs.

The specificity of microbiological inhibitor test (Table 1) is generally optimal in goat's milk with values over 95%. However, in individual samples of sheep milk, specificity was lower (90-92%) indicating a high percentage of "false-positives". In a previous study (Molina et al., 2003b) done by our team also in individual milk samples from sheep obtained during the entire lactation period, the specificity for BRT and Delvotest was higher (96 and 98% respectively). It is noteworthy 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).

Another disadvantage of these methods is that some factors may interfere in the response, such as the use of some preservatives for milk samples. When ewe's milk contains a preservative, i.e. acidiol, the specificity of methods diminishes (Molina et al., 2003b; Montero et al., 2005), although these "interferences" can be reduced by prolonging test incubation time (Molina et al., 1999; Montero et al., 2005). Even the matrix can affect the response of methods. In some of them, fatty acid contents have been detected (such as butyric and myristoleic acid) which can influence the test response (Romero, 2015), but do not appear to interfere with the results in most of the main components such as fat and proteins (Beltrán et al., 2015a). High somatic cell content in ewe's milk (Beltrán et al., 2015a) can increase the number of non-compliant samples. Interferences due to natural inhibitors have also been described but can be avoided by a previous milk heat treatment at 82-85 ºC for ten minutes before analysing milk (Molina et al., 2003b; Romero, 2015; Yamaki et al., 2004).

On the other hand, it has found in sheep milk samples originating from commercial farms a positive correlation between microbial count and positive results in microbiological methods for antibiotic detection (Yamaki et al., 2006).

Also, the presence of substances in milk related to some farming practices may also lead to "false-positive" results in microbial inhibitor tests. Thus, the presence of colostrum in sheep (Beltrán et al., 2010) and also goat's milk (Romero et al., 2014a) may produce anomalous results. The *in vitro* presence of antiparasitic substances such as closantel or diazinon in goat's milk produced false-positive results (Romero et al, 2015). Besides, residues from detergents used to clean milking and milk storage facilities may interfere with the results in sheep and goat's milk. Residues from alkaline detergents at concentrations greater than 2 ml/l and some domestic detergents (based on sodium laureth sulphate and ethanol) at concentrations above 1 ml/l could cause “false-positives” in sheep (Beltrán et al., 2009) and goat’s milk (Romero et al., 2014b, 2016).

With regard to receptor-based tests (rapid methods), Beltrán et al. (2013, 2014a, 2014b) studied the characteristics of several specific methods (Charm MRL BLTET, Betastar Combo, SNAP Betalactam, SNAP Tetracycline and TwinsensorBT) to analyse beta-lactams and/or tetracyclines in sheep’s and goat’s milk (Table 2). The table shows that most substances detected were equal to or below MRLs, presenting also a high specificity for individual samples from sheep and goats with the exception of the Twinsensor test in which false-positives results incremented notably in the case of milk produced at the end of the lactation period. Specificity was, however, very high when bulk milk was analysed

Beltrán et al. (2013, 2014a, 2014b) also observed that these tests showed neither cross-reactions with other antibiotic families nor interferences as a result of acidiol used as a preservative of milk samples.

Spanish legislation establishes the control of the presence of antibiotic residues in sheep and goat’s milk using screening methods that detect, at least, beta-lactam drugs. The detection rates of screening tests routinely used in Spain have been calculated (Beltrán et al., 2015b). In both types of milk, the simultaneous use of two screening tests with a different analytical basis (microbiological methods and receptor-based test) reached values close to 90% of the substances used in Spain However, antibiotics such as quinolones, macrolides or aminoglycosides are hard to detect unless they are present in milk at high levels. Thus, periodically controlling the presence of these substances by other methods is recommended to ensure the safety of milk and dairy products from sheep and goats.

2. PRESENCE OF CONTAMINANTS IN SHEEP AND GOAT’S MILK

The following milk contaminants must be monitored: organochlorides, including polychlorobiphenyls (PCB), dioxins, organophosphorous compounds, chemical elements (chrome, cadmium, lead, arsenic or mercury, among others) and mycotoxins. Many reach milk through food from contaminated crops, or from food stored with these crops, consumed by cattle. The main characteristic of such substances is that they largely accumulate in dairy products (cheese, cream, etc.) rather than in milk as they form complexes with proteins or accumulate in fat. Different cases have been described in which the presence of contaminants (lead, cadmium, arsenic, organochloride pesticides, PCBs, dioxins) in the milk of small ruminants and their derivate products has been detected, but the levels in most cases did not exceed the maximum limits allowed by law (Brambilla et al., 2011; Licata et al., 2012).

Concern about milk contaminated by mycotoxins, especially by aflatoxin M1, has grown in recent years, possibly due to the crises in Italy (2003) and Spain (2013), where large amounts of cereals were affected by and led to an emergency situation, during which milk was contaminated (Caravaca, 2013; Petri & Piva, 2007).

Apart from residues of antibiotics, aflatoxins are the most searched compounds in milk controls.

2.1. Mycotoxin contamination in milk

Mycotoxins are secondary metabolites caused by filamentous fungi which are associated with the appearance of adverse effects in humans and other vertebrates. Under certain conditions, some fungi like *Aspergillus*, *Fusarium* and *Penicillium* (Soriano, 2007) are capable of producing these compounds and contaminate food.

The most relevant mycotoxins in milk are aflatoxins (AFs). Eighteen types of AFs are known, of which AF B1, and its metabolic derivate AF M1, are the most harmful given their toxicity. AF M1 is excreted mainly by milk and has been studied in depth (Flores-Flores et al., 2015). Also, it is the only one that the EU has set MRLs for in milk (0.050 μg/kg).

The substrates most preferred by fungi are cereals and their derivates (Duarte et al., 2012; Soriano, 2007), and contamination may occur at any stage of the production process. When dairy cattle eat feed contaminated by mycotoxins, they may reach their milk or could metabolise to other toxins that can be excreted. A classic case is an animal that eats feed contaminated by mould of the genus *Aspergillus,* which has been reported to produce aflatoxin B1. The animal hepatically metabolises this toxin into aflatoxin M1, which is excreted through milk during the 12-24 h following intake, and if not detected, contamination could spread to dairy products (Battacone et al., 2003, 2012; Rubio, 2011). The transfer level is variable, between 0.08 and 6% depending on the species or on the animal's lactation level (Rubio, 2011). A recent review on the AF M1 incidence in sheep and goat's milk in Europe and Asia has indicated that it might be above 30% (Flores-Flores et al., 2015), although very few samples had a level that exceeded the European MRL. In 407 tanks, 82 silos and goat's milk curd in Spain (Rubio et al., 2011), the presence of AF M1 was detected in almost 50% of all samples, although levels were above the MRLs in only 0.7% of the tank samples and 1.2% of the silo samples.

2.2. Prevention and control of mycotoxins contamination in milk

Taking prevention measure is the best policy to avoid the contamination of milk with mycotoxins, relatively stable substances to the treatments applied in the dairy industry (Duarte et al., 2012; Rubio, 2011). There are not only control programmes that set MRLs, but also mycotoxin follow up programmes for sensitive products (animal feed), or even decontamination procedures (Rubio, 2011).

The key to prevention is avoiding mould growth on cereals and animal food to be fed to dairy cattle, for which there are three levels of action: reducing the risk of infecting crops (fungicides, controlled irrigation) by minimising crop susceptibility to fungal infections (varieties that resist mould growth, biological control); ensuring that transport and storage conditions do not allow fungal growth; training in AFs. If contamination cannot be prevented, applying detoxifying treatments at later stages is an option. It is recommendable to select those that destroy fungal spores which could synthesise the toxin, or destroy the toxin, but which must not produce or leave residues during treatment, nor alter the product’s nutritional value or its technological properties.

As aflatoxins are very harmful, the presence of these substances in milk and dairy products must be monitored and controlled. The EU set the MRL of AF M1 at 0.05 µg/kg, and so do several countries for cheese (0.2-0.45 µg/kg) and butter (0.02 µg/kg). European regulations also control sampling and analytical methodologies by CE Regulation 401/2006. Although the reference method in cow’s milk is HPLC with fluorometric detection, a wide variety of commercial methods based on ELISA immunoenzymatic assays have been developed in recent years. These tests have been studied in goat’s milk (Virdis et al., 2008) and sheep milk (Rubio et al., 2009) as well, and have been suggested to be an acceptable commercial option for AF M1 screening in shorter times than those needed for chromatography, which is used for official controls, and to confirm positive or doubtful results.

3. CONCLUSIONS

The presence of residues of antimicrobials and mycotoxins in milk and the strategy used for their detection is dynamic and changes along time. 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. All this makes the concept of an analytical strategy, as well as the periodical review and updating of an established strategy necessary, depending on the situation of each region or country, using the methodologies available and within to the existing legal framework.

With the implementation of a proper analytical strategy that prevents antibiotic residues and contaminants from reaching the food chain, the main objective of food safety is achieved.

Moreover, in general, by applying codes of good practices in the sheep and goat's milk production, the presence of antimicrobials residues and mycotoxins could be avoided. Thus, if suitable livestock management practices such as the identification of animals, the use of recommended cleaning products for milking and refrigeration equipment, the correct administration of drug treatments and a proper storage conditions of animal feed are followed by farmers, the risk of the presence of residues and contaminants in milk can be greatly reduced.

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Figure 1. Classification of antibiotics detection methods in milk.

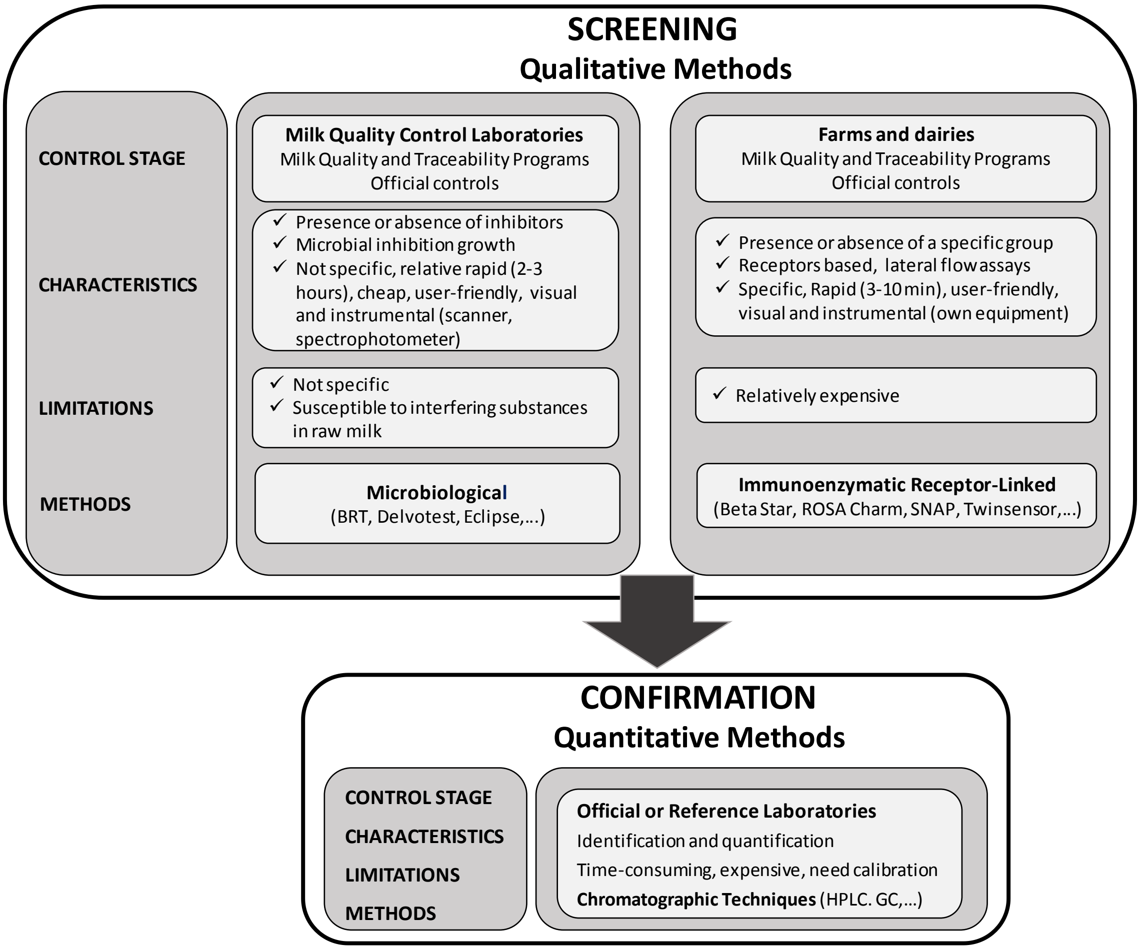

Table 1. Performance of microbiological screening methods widely used in Spain for the detection of antimicrobials in sheep and goat milk.

Principle	***Microbiological screening methods*** *Detection of bacterial growth inhibition (Geobacillus stereatermophilus var calidolactis) by a color indicator system (acid basic o redox).*							
Methodss	***BRT MRL***		***Delvotest MCS SP-NT***		***Delvotest MCS DA***		***Eclipse 100***	
Detection capability (MRL)[1]	*Sheep*	*Goat*	*Sheep*	*Goat*	*Sheep*	*Goat*	*Sheep*	*Goat*
Beta-lactams	73.3	73.3	86.7	80	86.7	80	86.7	80
Tetracyclines	×	×	×	×	×	×	×	×
Macrolides	66.7	66.7	33.3	33.3	33.3	33.3	33.3	33.3
Aminoglycosides	×	×	×	×	×	×	×	×
Quinolones	×	×	×	×	×	×	×	×
Sulphonamides	33.3	33.3	66.7	66.7	66.7	66.7	66.7	66.7
Specificiy[2]	95.2	98.6	92	96.9	90	95.7	90.4	99.4

[1] Beltran et al (2015a): Percentage of molecules detected at MRL equivalent concentration from each antibiotic family (Beta-lactams: Amoxicillin, ampicillin, penicillin, cloxacillin, dicloxacillin, nafcilin, oxacillin, cefacetrile, cefalonium, cefapirin, cefazolin, cefoperazone, cefquinome, ceftiofur and cephalexin; Tetracyclines: Chlortetracycline, oxytetracycline and tetracycline; Macrolides: Erythromycin, lincomycin and tylosin; Aminoglycosides: Gentamicin, neomycin and streptomycin; Quinolones: Enrofloxacin, ciprofloxacin and marbofloxacin; and Sulphonamides: Sulfadiazine, sulfadimethoxine and sulfametazine);×: CCβ > MRL from each antibiotic; [2]Specificity: Percentage of compliant outcomes in sheep (n=250) and goat (n=350) individual milk samples obtained during the entire lactation.

Table 2. Performance of receptor-binding methods widely used in Spain for the detection of antibiotics in sheep and goat's milk.

Principle	***Receptor-binding methods*** Detection of molecular interactions between antibiotics and ligands (receptor protein)							
Tests	***Charm MRL BLTET*** [1]		***BetaStar Combo*** [2,3]		***SNAP*** [2,3*]		***TwinsensorBT*** [2,3]	
	Sheep	*Goat*	*Sheep*	*Goat*	*Sheep*	*Goat*	*Sheep*	*Goat*
Beta-lactams								
Detection capability (MRL) [4]	86.7	86.7	86.7	86.7	100	100	86.7	86.7
Specificity individual milk samples	99.2	97.9	100	99	96.8	96.6	86.4	86.6
Specificity bulk milk samples	-	-	100	100	99	100	100	100
Tetraciclynes								
Detection capability (MRL)	100	100	100	100	100	100	100	100
Specificity individual milk samples	100	100	98.8	99	100	100	100	99
Specificity bulk milk samples	-	-	100	100	100	100	100	99

[1] Beltrán et al (2013); [2] Beltrán et al (2014a); [3] Beltrán et al (2014 b); [4]Detection capability (MRL): Percentage of molecules detected at MRL equivalent concentration from each antibiotic family (Beta-lactams: amoxicillin, ampicillin, benzylpenicillin, cloxacillin, dicloxacillin, nafcilin, oxacillin, cefacetrile, cefalonium, cefapirin, cefazolin, cefoperazone, cefquinome, ceftiofur and cephalexine; Tetracyclines: chlortetracycline, oxytetracycline and tetracycline); Specificity: Percentage of compliant outcomes in individual and bulk milk samples.* SNAP: different test for beta-lactam and tetraciclynes.