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

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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 $\mu g.Kg^{-1}$), ampicillin (4 $\mu g.Kg^{-1}$), benzylpenicillin (≤ 2 $\mu g.Kg^{-1}$), dicloxacillin (30 $\mu g.Kg^{-1}$), oxacillin (30 $\mu g.Kg^{-1}$), cefacetrile (≤ 63 $\mu g.Kg^{-1}$), cefalonium (≤ 10 $\mu g.Kg^{-1}$), cefapirin (≤ 30 $\mu g.Kg^{-1}$), desacetylcefapirin (≤ 30 $\mu g.Kg^{-1}$), cefazolin (≤ 25 $\mu g.Kg^{-1}$), cefoperazone (≤ 25 $\mu g.Kg^{-1}$), cefquinome (20 $\mu g.Kg^{-1}$), ceftiofur (≤ 50 $\mu g.Kg^{-1}$), desfuroylceftiofur (≤ 50 $\mu g.Kg^{-1}$) and cephalexin (≤ 50 $\mu g.Kg^{-1}$). However, this test could neither detect cloxacillin nor nafcillin at or below EU-MRL (CCβ > 30 $\mu g.Kg^{-1}$). The CCβ for tetracyclines was also

lower than EU-MRL for chlortetracycline (ewe's milk: ≤ 50 µg.Kg^{-1} and goat's milk: 75 µg.Kg^{-1}), oxytetracycline (≤ 50 µg.Kg^{-1}) and tetracycline (≤ 50 µg.Kg^{-1}). 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^{-1} and goat's milk: ≤ 50 µg.Kg^{-1}). Acidiol 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.

Keywords: ewe and goat milk, antibiotic, receptor binding assay, ROSA Charm

INTRODUCTION

In dairy ewes 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).

MATERIAL AND METHODS

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.

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, Hillerød, Denmark) to determine their chemical composition (fat, protein and total solids); SCC (somatic cell count) was obtained using Fossomatic 5000 (Foss, Hillerød, Denmark); BC (bacterial count) was determined using Bactoscan FC (Foss, Hillerød, 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).

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 < $300x10^3$ cell.mL^{-1} and bacterial count was < 10^5 cfu.mL^{-1}. 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 $x10^3$ cell.mL^{-1}, and bacterial count was < 10^5 cfu.mL^{-1}. 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

175 Community Reference Laboratories Residues (CRLs, 2010). This guideline document
176 supplements Commission Decision 2002/657/EC, and defines CCβ as the concentration
177 at which only ≤ 5 % false compliant results remain. For authorized analytes, the
178 concentration at which a screening test categorizes the sample as "screen positive"
179 (potentially non-compliant) and triggers a confirmatory test is called Screening Target
180 Concentration (STC) and it must be at or below EU-MRL. If the STC is set at half EU-
181 MRL, the occurrence of one or no false-compliant results following the analysis of at
182 least 20 "screen positive" control samples is sufficient to demonstrate that CCβ is below
183 EU-MRL and below or equal to 50 % of EU-MRL. If STC is set between 50 % and 90
184 % of EU-MRL, at least 40 "screen positive" control samples with no more than 2 false-
185 non compliant results will be sufficient to demonstrate that CCβ is below EU-MRL. If
186 STC approaches EU-MRL (below 10 % of EU-MRL) a maximum of 60 replicates with
187 no more than 3 false-non compliant results is required to demonstrate that CCβ is fit for
188 this purpose. Antibiotic concentrations used for the calculation of the CCβ of the Charm
189 MRL BLTET test were initially 0.5xEU-MRL (20 replicates); 0.75xEU-MRL (40
190 replicates) and 1xEU-MRL (60 replicates), respectively, only when necessary.

191 *Effect of preservative acidiol*

192 To evaluate the effect of the preservative acidiol on the response of the Charm MRL
193 BLTET test, antibiotic-free milk samples from 25 ewes and 25 goats were used.
194 Individual milk samples were divided into two aliquots; one without preservative and
195 one with acidiol; and analyzed by the Charm MRL BLTET test. Thereafter, each milk
196 sample was spiked with benzylpenicillin and oxytetracycline at EU-MRL (4 $\mu g.Kg^{-1}$
197 and 100 $\mu g.Kg^{-1}$, respectively) and analyzed again by the Charm MRL BLTET test.
198 Acidiol was prepared and used according to the Spanish regulation (Real Decreto
199 752/2011) which stipulates the composition (0.75 g chloramphenicol, 10 mL ethanol, 18

200 g sodium azide, 45 g trisodium citrate $5.5H_2O$, 0.35 g bromophenol blue, in 1000 mL of
201 distilled water) and the dosage of this preservative in ewe's and goat's milk (133 µl per
202 40 ml of raw milk).

203 *Antibiotics and spiked milk samples*

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

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

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

213 *Test procedure*

214 The Charm MRL BLTET test (Charm Sciences, Inc., Lawrence, MA) was employed
215 following the manufacturer's instructions. For ewes and goats, 300 µl of milk sample
216 was mixed with 300 µl of the dilution buffer (Sheep milk dilution buffer or Goat milk
217 dilution buffer, respectively. Charm Sciences, Inc.) and refrigerated for 10 minutes.
218 Thereafter, 300 µL of the mixture were placed in the sample compartment of the strip
219 placed in the ROSA Incubator (Charm Sciences, Inc.). The incubation time was set at
220 56 ºC for 16 minutes (two sets of 8 minutes), and results were interpreted visually by
221 three trained laboratory technicians and with the ROSA® Reader (ROSA® Pearl Reader.
222 Charm Sciences, Inc.).

223 The Charm MRL BLTET test uses receptors that bind beta-lactam and tetracycline
224 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.

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 $\mu g.Kg^{-1}$ and oxytetracycline: 100 $\mu g.Kg^{-1}$; 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.

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).

RESULTS AND DISCUSSION

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).

249 According to the instrumental interpretation (Table 3), specificity of the Charm MRL
250 BLTET test with adapted assay procedure for the detection of beta-lactam antibiotics
251 (BL line) was 99.2 % for ewes' milk (a false-positive rate of 0.8 %) and 97.9 % for
252 goats' milk (a false-positive rate of 2.1 %). Specificity was 100 % for the detection of
253 tetracyclines (TET line) in ewes' and goats' milk (no false-positive results). In all cases,
254 the specificity calculated according to the visual interpretation of the results was slightly
255 lower than that obtained by the ROSA® Reader, but no statistically significant
256 differences were found ($p > 0.05$).
257 Specificity results obtained in this study were optimal for both types of milk and
258 indicate that the characteristics of the milk do not influence the test response. The few
259 goat's milk samples that were classified as positive (7 false-positive results) had
260 standard characteristics of the Murciano-Granadina breed. The mean values for the
261 quality parameters considered were: pH: 6.73, fat: 6.47 %, protein: 4.12 %, total solids:
262 16.04 %, SCC: $519x10^3$ cell.mL^{-1} and BC: $62x10^3$ cfu.mL^{-1}.
263 There is only a limited number of evaluation studies of receptor binding assays in ewe's
264 and goat's milk available. Reybroeck et al. (2010) for the Betastar (1+1) test (Neogen
265 Corporation, Lansing, MI) obtained a specificity of 96.8 % for ewes' milk (1 out of 31
266 antibiotic-free milk samples) and 96.5 % for goats' milk (1 out of 29). The same result
267 (96.7 %) was obtained by Zeng et al. (1998) for the SNAP Betalactam test (IDEXX
268 Laboratories, Westbrook, ME) using raw commingled goats' milk (1 out of 30).
269 Comparing our results with those reported by other authors with different receptor
270 binding assays from Charm Sciences, Inc. (Lawrence, MA), Berruga et al. (2009) using
271 the Charm MRL BLTET test in ewe's milk obtained a lower specificity for the
272 detection of beta-lactam antibiotics (90 %) and a similar specificity (99 %) for
273 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.

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^{-1}), cefacetrile (≤ 63 μg.Kg^{-1}), cefalonium (≤ 10 μg.Kg^{-1}), cefapirin (≤ 30 μg.Kg^{-1}), desacetylcefapirin (≤ 30 μg.Kg^{-1}), cefazolin (≤ 25 μg.Kg^{-1}), cefoperazone (≤ 25 μg.Kg^{-1}), ceftiofur (≤ 50 μg.Kg^{-1}), desfuroylceftiofur (≤ 50 μg.Kg^{-1}) and cephalexin (≤ 50 μg.Kg^{-1}). For amoxicillin (4 μg.Kg^{-1}), ampicillin (4 μg.Kg^{-1}), dicloxacillin (30 μg.Kg^{-1}), oxacillin (30 μg.Kg^{-1}) and cefquinome (20 μg.Kg^{-1}) 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^{-1}).

The CCβ for tetracyclines was also lower than EU-MRL for chlortetracycline (ewe's milk: ≤ 50 μg.Kg^{-1} and goat's milk: 75 μg.Kg^{-1}), oxytetracycline (≤ 50 μg.Kg^{-1}) and tetracycline (≤ 50 μg.Kg^{-1}). 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^{-1} and goat's milk: ≤ 50 μg.Kg^{-1}). For 4-epichlortetracycline and 4-epitetracycline the CCβs were above EU-MRL (CCβ > 100 μg.Kg^{-1}).

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^{-1}). 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).

Effect of acidiol on the test response

The presence of acidiol 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 acidiol 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 $\mu g.Kg^{-1}$) nor with oxytetracycline (100 $\mu g.Kg^{-1}$) no matter whether the interpretation of the results was made visually or instrumentally.

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 acidiol in milk

samples, which also allows its use in milk quality control laboratories which normally analyze ewe's and goat's milk with acidiol.

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.

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. Acidiol 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.

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REFERENCES

Althaus, R.L., A. Torres, C. Peris, M.C. Beltrán, N. Fernández, and M.P. Molina. 2003. Accuracy of BRT and Delvotest microbial inhibition tests as affected by composition of ewe's milk. J. Food Prot., 66:473-478.

Berruga, M.I., M. Roca, M.P. Molina, and A. Molina. 2009. Evaluation of receptor-based tests for the detection of penicillins and tetracyclines residues in ewe milk. IDF World Dairy Summit. Berlin, Germany.

Berruga, M.I., M.C. Beltrán, B. Novés, A. Molina, and M.P. Molina. 2011. Effect of penicillins on the acidification of yogurt made from ewe's milk during the storage. Pages 145-149 in Science and Technology Against Microbial Pathogens. Research, Development and Evaluation. Ed. Mendez-Vilas, A. World Scientific Publishing. Hackensack, NJ.

Charm, S.E., and E. Zomer. 1995. The evolution and direction of rapid detection/identification of antimicrobial drug residues. Pages 224-233 in Proc. Residues of antimicrobial drugs and other inhibitors in milk. International Dairy Federation Special Issue 9505. Int. Dairy Fed., Brussels, Belgium.

Community Reference Laboratories Residues (CRLs). 2010. Guidelines for the validation of screening methods for residues of veterinary medicines. Available at http://ec.europa.eu/food/food/chemicalsafety/residues/lab_analysis_en.htm (Last update: 20-01-2010).

396 Contreras, A., M.J. Paape, A.L. Di Carlo, R.H. Miller, and P. Rainard. 1997. Evaluation
397 of selected antibiotic residue screening tests for milk from individual goats. J. Dairy
398 Sci., 80:1113-1118.
399 European Union. 2002. Commission Decision 2002/657/EC of 12 August 2002
400 implementing Council Directive 96/23/EC concerning the performance of analytical
401 methods and the interpretation of results. Off. J Eur Commun 2002; L 221:8-36.
402 European Union. 2009. Regulation (EC) Nº 470/2009 of 6 May 2009 laying down
403 Community procedures for the establishment of residue limits of pharmacologically
404 active substances in foodstuffs of animal origin, repealing Council Regulation
405 (EEC) Nº 2377/90 and amending Directive 2001/82/EC of the European Parliament
406 and of the Council and Regulation (EC) Nº 726/2004 of the European Parliament
407 and of the Council. Off: J. L 152:11-22.
408 European Union. 2010. Regulation (EU) Nº 37/2010 of 22 December 2009 on
409 pharmacologically active substances and their classification regarding maximum
410 residue limits in foodstuffs of animal origin. Off. J. L 15:1-72.
411 Food and Drug Administration Center for Veterinary medicine (FDA). 2005. MI-05-05:
412 Tolerance and/or Safe Levels of animal drug residues in milk. Available at
413 http://www.fda.gov/Food/FoodSafety/Product-
414 SpecificInformation/MilkSafety/CodedMemoranda/MemorandaofInformation/ucm0
415 77350.htm (Last update: 14-11-2011).
416 Haenlein, G.F.W. 2001. Past, present and future perspectives of small ruminant dairy
417 research. J. Dairy Sci., 84:2097-2115.
418 International Dairy Federation (IDF). 2002. Guidelines for the standardized description
419 of immuno- or receptor assays for the detection of antimicrobial residues. IDF
420 Standard Nº188:2002. Int. Dairy Fed., Brussels, Belgium.

International Dairy Federation (IDF). 2010. Current situation & compilation of commercially available screening methods for the detection of inhibitors/antibiotic residues in milk. Bulletin of the International Dairy Federation 442. Int. Dairy Fed., Brussels, Belgium.

Molina, A., M.P. Molina, R.L. Althaus, and L. Gallego. 2003a. Residue persistence in sheep milk following antibiotic therapy. Vet. J., 165:84-89.

Molina, M.P., R.L. Althaus, S. Balasch, A. Torres, C. Peris, and N. Fernández. 2003b. Evaluation of screening test for detection of antimicrobial residues in ewe milk. J. Dairy Sci., 86:1947-1952.

Packham, W., M.C. Broome, G.K.Y. Limsowtin, and H. Roginski. 2001. Limitations of standard antibiotic screening assays when applied to milk for cheesemaking. Aust. J. Dairy Technol., 56:15–18.

Pengov, A., and A. Kirbis. 2009. Risks of antibiotic residues in milk following intramammary and intramuscular treatments in dairy sheep. Anal. Chim. Acta, 637:13-17.

Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother., 53:28-52.

Real Decreto Nº 752/2011 de 27 de mayo, por el que se establece la normativa básica de control que deben cumplir los agentes del sector de leche cruda de oveja y cabra. BOE, 9 de Junio de 2011, 137:58609-58630.

Requena, R., S. Balasch, C. Peris, M. Rodríguez, and N. Fernández. 2010. Dose response of lactating dairy ewes during suckling and milking to bovine somatotropin. J. Anim. Sci., 88:3136-3144.

446 Reybroeck, W., S. Ooghe, H.F. De Brabander, and E. Daeseleire. 2010. Validation of
447 the Beta-s.t.a.r 1+1 for rapid screening of residue of β-lactam antibiotics in milk.
448 Food Addit. Contam. 27:1084-1095.
449 Reybroeck, W., S. Ooghe, H.F. De Brabander, and E. Daeseleire. 2011. Validation of
450 the Charm MRL-3 for fast screening of β-Lactam antibiotics in raw milk. J. AOAC
451 Int., 94:373-382.
452 Salama, A.A.K., X. Such, G. Caja, M. Rovai, R. Casals, E. Albanell, M.P. Marín, and
453 A. Martí. 2003. Effects of once versus twice daily milking throughout lactation on
454 milk yield and milk composition in dairy goats. J. Dairy Sci., 86:1673-1680.
455 Sanders, P., A. Bousquet-Melou, C. Chauvin, and P.L. Toutain. 2011. Utilisation des
456 antibiotiques en élevage et enjeux de santé publique. INRA Prod. Anim., 24:199-
457 204.
458 Salter, R.S., D. Douglas, L. McRobbie, J. Quintana, D. Legg, J. Schwartz, D. Conaway,
459 C. McPhee, S. Saul, and R. Markovsky. 2011. Validation of the Charm 3 SL3 β-
460 Lactam test for the screening raw milk in compliance with the U.S. pasteurized milk
461 ordinance. J. AOAC Int., 94:348-357.
462 Zeng, S.S., S. Hart, E.N. Escobar, and K. Tesfai. 1998. Validation of antibiotic residue
463 tests for dairy goats. J. Food Prot., 61:344-349.

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_20
Nafcilin	Sigma-Aldrich	N3269	MeOH / H_20
Oxacillin	Sigma-Aldrich	46589	MeOH / H_20
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_20
Cephalexin	Sigma-Aldrich	C4895	H_2O
Lincosamides			
Lincomycin	Sigma-Aldrich	31727	H_2O
Macrolides			
Erythromycin	Sigma-Aldrich	E6376	EtOH / H_20
Polimyxins			
Colistin	Sigma-Aldrich	C4461	H_2O
Quinolones			
Enrofloxacin	Sigma-Aldrich	33699	AcOH 5% / H_20
Sulfonamides			
Sulfadiazine	Sigma-Aldrich	S6387	H_2O
Tetracyclines			
Chlortetracycline	Sigma-Aldrich	C4881	NaOH 0.1N / H_2O
4-epichlortetracycline	Acros[5]	268235000	MeOH / H_20
Oxytetracycline	Sigma-Aldrich	O4636	HCl 0.1N / H_2O
4-epioxytetracycline	Acros	25771	MeOH / H_20
Tetracycline	Sigma-Aldrich	T3258	HCl 0.1N / H_2O
4-epitetracycline	Acros	233125000	MeOH / H_20

[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

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

	Ewe's milk (n= 250)				Goat's milk (n= 350)			
Parameter	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] ($x10^3$ cfu.mL^{-1})	566	1,508	6	9,999	74	306	10	4,829
SCC[5] ($x10^3$ cell.mL^{-1})	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

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	BL	2	1	247	98.8	2	248	99.2
(n = 250)	TET	0	0	250	100	0	250	100
Goats	BL	7	2	341	97.4	7	343	97.9
(n = 350)	TET	0	1	349	99.7	0	350	100

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

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 ($\mu g.Kg^{-1}$)	STC[1] ($\mu g.Kg^{-1}$)	Positive/Total samples[2]	Positive Results (%)	CCβ ($\mu g.Kg^{-1}$)
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

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 ($\mu g.Kg^{-1}$)	STC[1] ($\mu g.Kg^{-1}$)	Positive/Total samples[2]	Positive Results (%)	CCβ ($\mu g.Kg^{-1}$)
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

Figure 1. Effect of acidiol in ewe's and goat's milk samples on the results of the Charm MRL BLTET test