International Dairy Journal 62 (2016) 39–42

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Short communication

Detection of antibiotics in goats' milk: Comparison of different commercial microbial inhibitor tests developed for the testing of cows' milk

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form
7 July 2016
Accepted 9 July 2016
Available online 21 July 2016

ABSTRACT

Nine microbial inhibitor tests validated for cows' milk (BR-AS Special, CMT-Copan Milk Test, Delvotest SP-NT, Delvotest T, Brilliant Black Reduction Test MRL, Charm Blue Yellow II, Charm CowSide II, Eclipse 100, Eclipse 3G) were applied to milk samples from 200 different individual goats. Interpretation of the results was based on visual and instrumental reading. Samples initially testing positive were retested and also tested after a milk pre-treatment (heat treatment, fat removal or fat removal followed by heat treatment). With instrumental reading, most microbial tests commonly used for bovine milk were suitable for goats' milk (specificity ≥95%), except for BR-AS Special, Charm Blue Yellow II and Delvotest SP-NT. However, visual reading of the results decreased the specificity, with ≥95% specificity only for CMT-Copan Milk Test, Eclipse 3G and Delvotest T. Fat removal followed by heat treatment proved the most appropriate milk treatment to reduce false positive results for almost all tests.

1. Introduction

Currently, antibiotic residues in milk are still of great concern to different sectors such as milk producers, the dairy industry, regulatory agencies and consumers. As milk production by small ruminants increased in recent years, the use of antibiotics in dairy goats has become a usual practice in veterinary medicine to treat mastitis and other diseases (Silanikove, Leitner, Merin, & Prosser, 2010).

The European Union established Maximum Residue Limits (MRLs) for veterinary medicinal products in Commission Regulation (EU) No 37/2010 (EU, 2010). Inhibitory substances in milk are routinely screened at farms, dairies and laboratories. Currently, several commercial methods to detect antibiotics are available (IDF, 2010). Microbial inhibitor tests are the most commonly used, because they are fast, easy to use, and relatively cheap and can detect a wide spectrum of compounds. Evaluating the performance of screening tests, requirements are stipulated for the rate of false compliant results. Following Commission Decision 2002/657/EC (EC, 2002) this rate should be <5% (β-error) at the level of interest (CCβ). In the same Commission Decision, as a general requirement for specificity, it is stated that a method should be able to distinguish between the analyte (antibiotic residue) and the other substances under the experimental conditions. Therefore, specificity is associated with the presence of false positive results and is of great interest to evaluate the analytical capacity of a test. But the legislation does not fix maximum levels for the rate of false positive results. A positive test result is considered to be false positive when no antibiotics are present in the milk. To determine false positive results, a large number of milk samples from animals not treated with veterinary medicinal products should be analysed.

Microbial inhibitor tests are not specific for antibiotic residues but may be affected by lactoferrin or lysozyme (Carlsson, Björck, & Persson, 1989), high somatic cell count (Andrew, 2001), abnormal fat content (Reybroeck & Ooghe, 2012) or preservatives (Molina et al., 2003) capable of inhibiting the growth of the test organism and contributing to false positive results. Inhibitor tests have been developed for the testing of cows' milk, but are also used for the analysis of milk from other species, such as goats. Most of studies about false non-compliant results were performed with cows' or ewes' milk (Althaus et al., 2003; Andrew, Frobish, Paape, & Maturin, 1997; Beltrán, Berruga, Molina, Althaus, & Molina, 2015; Kang, Jin,

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http://dx.doi.org/10.1016/j.idairyj.2016.07.004
0958-6946/

& Kondo, 2005; Molina et al., 2003) and hence limited information for goats' milk is available.

False positive results can have serious consequences, as producers and the dairy industry are encountered with economic losses. Validation of the tests for goats' milk is very important for the selection of the most appropriate testing strategy for a correct interpretation of the test results and to ensure good monitoring for antibiotics in dairy goats' milk.

The main objective of the study was to compare the performance of microbial inhibitor tests developed for the detection of antimicrobial residues in cows' milk, when used for goats' milk. A second objective was to reduce the number of false positive results by testing three milk treatments such as heating, fat removal or fat removal followed by heating.

2. Materials and methods

The experimental study was carried out in the Technology and Food Science unit (Melle, Belgium) of the Institute for Agricultural and Fisheries Research (ILVO-T&V).

2.1. Milk samples

Two hundred individual milk samples of different goats of White Saanen breed were collected from three Flemish goat farms. The sampling of milk of different individual goats was performed in the afternoon milking, around 5 and 6 p.m. Each sample of individual goats' milk was kept refrigerated at $\leq$4 °C until transport to the laboratory the next morning. After analysis the remaining milk was frozen at −30 °C in aliquots for additional residue analysis.

2.2. Microbial inhibitor tests

Milk samples were tested 14–20 h post-milking by means of nine different microbial inhibitor tests: BR-AS Special, CMT-Copan Milk Test, Delvotest SP-NT and Delvotest T from DSM Food Specialties (Delft, The Netherlands), Brilliant Black Reduction Test MRL (BRT MRL) from Analytik in Milch Produktions-und Vertriebs-GmbH (Munich, Germany), Charm Blue Yellow II and Charm CowSide II from Charm Sciences Inc. (Lawrence, MA, USA), Eclipse 100 and Eclipse 3G from ZEULAB S.L. (Zaragoza, Spain).

All tests are based on the inhibition of the growth of the microorganism *Geobacillus stearothermophilus* var. *calidolactis*. The colour indicator in most of the methods used is bromocresol purple, but for the BRT MRL and BR-AS Special it is brilliant black. Of all kits, the 96-well microtitre plate format was used, except for Charm CowSide II that was in individual test vials. The commercial tests were used following the instructions of the kit manufacturers. Milk samples were performed in duplicate and in every run of each inhibitor test control standards were included: blank reference milk: mixture of 6 negative goats' milk samples and antibiotic standards (oxytetracycline, O5875; benzylpenicillin, PENNA; sulphadiazine, S8626 and sulphadoxine, S7821 provided by Sigma–Aldrich, Bornem, Belgium).

All microbial tests were incubated in a covered waterbath (Type 19+MP thermostat from Julabo Labor-technic GmbH (Seelbach, Germany)) at 64.0 ± 0.2 °C, except for the Charm CowSide II test vials that were incubated in a Charm digital dry block incubator 220 V (Charm Sciences Inc.), Eclipse 100 and Eclipse 3G plates were incubated in a FX incubator (ZEULAB S.L) at 65 °C. The incubation time is different between the microbial methods employed (between 2 or 3 h). Some microbial tests as BRT MRL, Charm CowSide II, Charm Blue Yellow II, Delvotest SP-NT and Eclipse 3G required a longer incubation time (10–25 min) to obtain negative results for the reference blank milk controls on each plate, possibly because the indicated incubation times are set for cows' milk.

The interpretation of the results (colour of the test medium) was carried out visually and instrumentally, except for the Charm CowSide II test, which was only interpreted visually. The instrumental interpretation was carried out following the manufacturers' indications using the adequate equipment for each microbial inhibitor tests. For BR-AS Special, Delvotest SP-NT, Delvotest T, CMT and Charm Blue Yellow II a flatbed scanner and software programme is necessary to determine the results. However, BRT MRL, Eclipse 100 and Eclipse 3G results were interpreted photometrically using a spectrophotometer at different wavelength (nm). All microbial inhibitor tests with instrumental reading present a different cut-off established by the commercial company to discriminate between negative and positive results. It is worth noting that this cut-off was set for cows' milk. In this study the categories 'negative' and 'positive' were further split by calculating an extra border value for classification by subtracting and adding 3 × SD to the fixed cut-off value, respectively. Hence 4 categories were obtained: "−/−", "−/+", "+/−" and "+/+" where the categories "−/+" and "+/−" are containing the border line results. By visual interpretation of the colour at the end of the incubation the samples were also classified in 4 categories namely "−/−" (yellow), "−/+" (greenish, yellow–blue), "+/−" (light blue, blue–yellow) and "+/+" (blue to purple).

2.3. Treatments of positive milk samples

To check that all milk samples used in the study were free of antibiotic residues, the positive milk samples for any microbial inhibitor test were tested the next day with the addition of β-Lactamase ES (Sekisui Enzymes West Malling, UK), 4-aminobenzoic acid (PABA) (Sigma–Aldrich) or $CaCl_2$ (Merck KGaA, Darmstadt, Germany), respectively and by means of different group-specific receptor-binding assays (Twinsensor BT, 3SENSOR, and 4SENSOR from Unisensor s.a. (Liège, Belgium); Charm MRL BLTET2 from Charm Sciences Inc. and βeta-star from Neogen Corporation (Lansing, MI, USA). After the analyses by rapid tests, the positive samples were analysed with a chromatographic method (LC-MS/MS) at ILVO as described by Daeseleire, De Ruyck, and Van Renterghem (2000).

Milk samples testing positive in the initial residue screening, were retested after three different milk treatments to reduce the number of false positive results: (a) heating (80 °C for 10 min), (b) fat removal (centrifuging at 3100×*g* for 10 min at 4 °C, then removal of the fat on the top with cotton tipped applicators) and (c) fat removal followed by heating. Milk without any treatment was analysed to allow comparison.

2.4. Statistical analysis

The differences between the reading system used for the interpretation of the microbial tests results (visual and instrumental) were tested with McNemar's test. Statistical analyses were performed using SAS, version 9.2, 2001 (SAS Institute Inc., Cary, NC, USA). Samples showing questionable results of category "+/−" were recorded as positive results for statistical analysis.

3. Results and discussion

One positive sample, containing a low concentration of oxytetracycline (<10 μg kg^{-1}), confirmed by chromatographic analysis, was removed from the study (n = 199). Table 1 shows the specificity by the visual and instrumental reading of different commercial inhibitor tests developed for cows' milk for the remaining 199