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Residue concentration of cefquinome taking into account different milk fractions and comparing the performance of two screening tests

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Research Article

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Abstract

This Research Communication describes the residue concentration of a dry cow antibiotic in two different milk fractions and describes effects of milk fraction and milk composition on the test performance of a rapid screening and a microbial inhibitor test. Thirteen dry cows were treated with an intramammary dry cow antibiotic containing 150 mg cefquinome. Quarter foremilk and stripping samples were collected on the first 10 d postpartum. All milk samples were analyzed for milk composition by the local Dairy Herd Improvement Association and were tested for antibiotic residues using the rapid screening test Milchtest BL and the microbial inhibitor test Delvotest BR Brilliant Plates. The residue concentration of cefquinome was determined in foremilk and stripping samples from milkings 1, 2, 3, 5, and 7 after calving using high performance liquid chromatography – tandem mass spectrometry. The logarithm of cefquinome concentration (logCef) was higher in foremilk than in stripping samples and higher in milk samples with lower lactose content. Furthermore, logCef decreased with the number of milkings ($P < 0.001$). The Milchtest BL was more likely to be not evaluated (i.e. no test and control line or no control line appeared) in stripping samples and milk samples with higher protein content. In the Delvotest BR Brilliant Plates milk samples with higher protein content were more likely to have a false positive result (i.e. the screening test result was positive, but the HPLC-MS/MS result was below the detection limit of the screening test). These results indicate that foremilk is the recommended milk fraction to be tested for residues of cefquinome and that a high protein content can be a cause of test failure and false positive results when milk during the first 10 d postpartum is tested for antibiotic residues using screening tests.

Screening tests should be used regularly on farms to detect antibiotic residues in bulk tank milk as well as milk from individually treated cows to ensure food safety (Jones, 2009; IDF, 2014*b*). These screening tests, however, were only evaluated in the United States by the Federal Drug Administration (FDA) for use on raw commingled milk samples; there is no regulatory requirement for milk from individual cows to be tested (FDA, 1996). We expect that foremilk (hand collected milk before milking) and stripping samples (hand collected milk after milking) from individual cows are most commonly used for screening tests, because the sampling procedure in the milking parlour is easier than for composite milk samples. For an appropriate use of such screening tests, knowledge of the relationship and potential confounding between milk fraction (foremilk and strippings) and antibiotic concentration in milk samples from individual cows is important.

Stockler *et al.* (2009) detected more than twice as high cephapirin concentrations in foremilk than in bucket milk or strippings from lactating cows at the first milking after intramammary (IMM) infusion. They hypothesised that changes in milk composition among milk fractions might affect the distribution of the antibiotic within the udder (Stockler *et al.*, 2009). The highest logarithm of somatic cell count (logSCC) and fat content were detected by Vangroenweghe *et al.* (2002) in residual milk and the highest content of protein in foremilk, cisternal and main milk. We hypothesise that these changes in milk composition between the milk fractions might affect the concentration of antibiotic residues after dry cow treatment and the test characteristics of screening tests used.

Therefore, the objective of our study was (1) to investigate the concentration of residues after antibiotic dry cow treatment using a cefquinome product in two different milk fractions (foremilk or strippings) taking into account milk composition (fat, protein, lactose, urea and

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SCC) and (2) to evaluate the test characteristic of two commercially available screening tests in relationship to the milk fraction and the milk composition.

Materials and methods

Data collection

The study was conducted between November 2014 and May 2015 at the Clinic for Animal Reproduction (Freie Universität Berlin, Berlin, Germany). Thirteen healthy, dry Holstein-Friesian and Holstein-Friesian crossbreed dairy cows were enrolled in the study. Three quarters of each cow received a single treatment with an intramammary (IMM) dry cow antibiotic (150 mg cefquinome; Virbactan, Virbac Ltd., Carros, France), one quarter on each of d21, 14 and 7 before calculated calving date, which was 280 d after artificial insemination), respectively. One quarter of each cow received no treatment. Day of treatment was allocated to each quarter at random. This treatment scheme resulted from the fact that the enrolled cows were part of a larger study on antibiotic residues after short dry periods using a quarter-based approach. The cows were not dried off shortly before calving, but previously dried-off cows were selected and retreated with an IMM dry cow antibiotic following the above-described treatment scheme. This antibiotic treatment scheme falls into the category of extra-label drug usage, as all four quarters of a cow should be treated at the same time. All quarters were monitored before and once a day after treatment for signs of clinical mastitis (i.e. firmness, pain, heat, redness, swelling). After calving cows were milked twice daily at 07.00 and 19.00 h using a portable bucket milking machine.

Milk samples were collected on the first 10 d postpartum during milking times at 07.00 and 19.00 h (first to twenty-second milking after calving). Two samples (13 and 30 ml) each were collected from each quarter before (foremilk samples) and after milking (stripping samples), respectively. The foremilk samples were collected after examining the first two streams from each quarter for signs of clinical mastitis (i.e. clots, flakes) as well as subclinical mastitis using the California Mastitis Test. The stripping samples were collected immediately after removing the milking cluster. Antibiotic residues were tested with a lateral flow test Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany) with a detection limit of 20 µg/kg for cefquinome. Procedures were conducted according to the recommendations of the manufacturer. Test results were recorded as negative (i.e. the test line was darker than or as dark as the control line), positive (i.e. the test line was fainter than the control line or no test line but the control line appeared) and as not able to be evaluated (i.e. no test and control line or no control line appeared), respectively. Residue concentration of cefquinome in foremilk and stripping samples from the first, second, third, fifth and seventh milking after calving were determined using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with a limit of quantification of 1 ng/g and a limit of detection of 0.5 ng/g for cefquinome. The 30 ml foremilk and stripping samples were analysed for milk composition (fat, protein, lactose, urea, SCC) by trained technicians using the CombiFoss (Foss, Hilleroed, Denmark) at the local DHIA. Furthermore, all samples were tested for antibiotic residues using the microbial inhibitor test Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands) with an incubation time of 2 h 45 min and a detection limit of 100 µg/kg for cefquinome. The test was conducted and evaluated according to IDF 471/2014 (IDF, 2014*a*). Test results of the Milchtest BL and Delvotest BR Brilliant Plates from milkings 1, 2, 3, 5, and 7 after calving were compared with the HPLC-MS/MS results and were categorised as false positive when the test result was positive and the HPLC-MS/MS result below the detection limit of the screening test and as false negative when the test result was negative and the HPLC-MS/MS result above the detection limit of the screening tests.

Statistical analysis

Data were recorded in Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analyses performed with IBM SPSS Statistics (version 22.0; IBM Deutschland GmbH, Ehningen, Germany) and Medcalc (version 12.4.0.0, Mariakerke, Belgium). The cefquinome concentration and SCC were transformed to common logarithms to achieve normal distribution (logCef and logSCC). The effect of milk fraction (foremilk *v.* stripping samples), the number of milkings (first to twenty-second milking after calving), fat (%), protein (%), lactose (%) and urea content (mg/l) and logSCC on logCef were determined in a mixed-model ANOVA. The effect of milk fraction on the milk composition was also determined using a mixed-model ANOVA. Mixed models were built according to the model building strategies developed by Dohoo *et al.* (2009). The random effect of udder quarter within cow was included in all models. The number of milkings was considered as repeated factor.

To investigate potential associations between the milk fraction, fat, protein, lactose, urea content and logSCC and the test results 'not evaluated' and 'false positive' of Milchtest BL and 'false positive' of Delvotest BR Brilliant Plates binary logistic regressions were used. All values reported are LSM ± SEM. The significance level was set at $P \leq 0.05$.

Results and discussion

Residue concentration of cefquinome

No signs of subclinical or clinical mastitis were detected in any quarter throughout the study period, therefore, 2027 values of fat, protein and lactose, 2099 values of SCC and 1997 values of urea from foremilk and stripping samples were used in the final analysis. Milk composition differed between foremilk and stripping samples ($P < 0.001$), with higher protein, lactose and urea content in foremilk samples and higher fat content and logSCC in stripping samples (online Supplementary Table S1).

One hundred and eighty-four foremilk and 167 stripping samples from milkings 1, 2, 3, 5, and 7 after calving were analysed for the residue concentration of cefquinome using HPLC-MS/MS. The logarithm of cefquinome concentration (logCef) was affected by the milk fraction ($P = 0.001$), the milking ($P < 0.001$) and the lactose content of the milk sample ($P < 0.001$). The logCef was higher in foremilk (1.495 ± 0.080) compared with stripping samples (1.377 ± 0.078; $P = 0.001$). After having retransformed the values of logCef for better comparability, the residue concentration of cefquinome was 31.26 and 23.82 ng/g in foremilk and stripping samples, respectively. Our results agree with Stockler *et al.* (2009) for a lactating cow antibiotic containing 200 mg cephapirin sodium. They detected 44.2 µg/ml cephapirin in foremilk, 18.5 µg/ml in stripping samples and 15.7 µg/ml in bucket milk at the first milking following the IMM antibiotic treatment.

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Table 1. Association between the evaluability of the Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany) and milk fraction and milk composition ($n = 303$ milk samples)

Variable	Estimate	SE	*P*-value	Odds ratio	CI for Odds ratio
Milk fraction foremilk strippings	3.647	0.333	< 0.001 reference	38.374	19.973–73.728
Protein	−0.553	0.068	< 0.001	0.575	0.504–0.657
LogSCC	−0.290	0.168	0.084	0.748	0.538–1.040

Table 2. Association between false positive results of the Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands) and the milk composition ($n = 47$ milk samples)

Variable	Estimate	SE	*P*-value	Odds ratio	CI for Odds ratio
Protein	−0.212	0.043	< 0.001	0.809	0.744–0.880
Urea	0.006	0.003	0.055	1.006	1.000–1.012

While the cephapirin concentration in the study by Stockler *et al.* (2009) was 2.39 times higher in foremilk than in stripping samples, in our study the cefquinome concentration in foremilk was only 1.46 times higher. This difference could be explained by the longer exposure time of the drug in the udder, which might translate to more intense distribution within the udder. The value of logCef decreased with the number of milking ($P < 0.001$) and additionally, an interaction between the milk fraction and the number of milking was detected ($P = 0.001$; Supplementary Fig. S1). This is consistent with residue depletion studies in lactating cows which have detected high concentrations of cefquinome in milk at the first milking after the last administration and concentrations under the maximum residue limit at the 10th milking (CVMP, 1995). The value of logCef was higher in samples with lower lactose content ($P < 0.001$). This was the only effect of milk composition on the cefquinome concentration detected in our study and the mechanism remains unclear.

Screening tests

Three hundred and eighty-three foremilk and 448 stripping samples were tested for residues of cefquinome using the rapid screening test Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany). Three hundred and three test results (82.8% of stripping samples, 17.2% of foremilk samples) of the Milchtest BL were not evaluated because no test or control line appeared. Foremilk samples had 38.4 times higher odds to be evaluated in the Milchtest BL than tests of stripping samples (Table 1). A high protein content significantly increased the odds of non-evaluated results in the Milchtest BL (Table 1), whilst a high logSCC was associated with an arithmetic (non-significant) increase.

The higher proportion of evaluated foremilk samples might be related to the lower fat content of foremilk compared with stripping samples. Andrew (2000) hypothesised that high milk fat may hinder the milk movement along the gradient in the CITE Snap assay and result in a test failure and false-positive outcomes. In our study the milk samples did not flow far enough through the membrane to reach the test and control line. High fat and protein content cause a higher dynamic viscosity of milk (Alcântara *et al.*, 2012) probably because the high viscosity of early lactation milk impedes the flow of the milk samples through the membrane of the Milchtest BL. A high logSCC might also obstruct milk flow through the test membrane.

Corresponding results of HPLC-MS/MS for residue concentrations of cefquinome were available for 68 test results of the Milchtest BL and they were used as a gold standard. Nineteen test results of the Milchtest BL were false positive (7.6 ± 1.1 ng/g; range: 0.5 to 18.40 ng/g) and no test result was false negative. False positive test results of the Milchtest BL were neither associated with the milk fraction nor milk composition ($P > 0.100$).

Seven hundred and seventy-three foremilk and 775 stripping samples were tested at the local DHIA using the Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands). Corresponding HPLC-MS/MS results for residue concentrations of cefquinome were available for 348 test results of the Delvotest BR Brilliant Plates and they were used as a gold standard again. Forty-seven test results of the Delvotest BR Brilliant Plates were false positive (33.8 ± 4.7 ng/g; range: 0.5 to 98.85 ng/g) and six were false negative (180.5 ± 51.2 ng/g; range: 113.03 to 435.11 ng/g). Milk samples with higher protein content were more likely to have a false positive result in the Delvotest BR Brilliant Plates (Table 2) and those with a lower urea content showed a similar arithmetic association (non-significant). Natural inhibitors are known to be elevated in colostrum and mastitic milk (Hillerton *et al.*, 1999). They could be the reason for the correlation between increased protein content and false positive results of the Delvotest BR Brilliant Plates in our study, as the natural inhibitors might inhibit the growth of the test organism *Bacillus stearothermophilus*. Kang *et al.* (2005) collected 73 foremilk samples from lactating cows with clinical mastitis after withdrawal times (2 to 5 d) from intramammary antibiotic treatment (beta-lactams, tetracyclines, sulfonamides and aminoglycosides) and tested with the Delvotest SP for antibiotic residues. As 21 from 24 positive results were negative after being heated at 82 °C for 5 min, the author concluded that these 21 positive results were caused by natural inhibitors in the milk samples, which were inactivated by the heat treatment. The weak correlation between increased urea content and correct results of the Delvotest BR Brilliant Plates remains unclear and to our knowledge no other study reported a comparable correlation. Milk fraction ($P = 0.799$), fat ($P = 0.887$) and lactose content ($P = 0.110$) as well as logSCC ($P = 0.453$) had no effect on the correctness of the results in the Delvotest BR Brilliant Plates.

The results of this study indicate that foremilk should be the recommended milk fraction to be tested for residues of cefquinome and to be used in lateral flow tests to avoid test failure. Furthermore, high protein content should be considered as a

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cause of test failure and false positive results when milk during the first 10d postpartum is tested for antibiotic residues using lateral flow or microbial inhibitor tests.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000554.

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