Elimination of selected mastitis pathogens during the dry period

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ABSTRACT

We aimed to evaluate the elimination of 4 different mastitis pathogens, Strep. agalactiae, Mycoplasma bovis, Staph. aureus, and Strep. uberis, from infected udder quarters during the dry period using quantitative PCR. The second purpose of this study was to evaluate the association between milk haptoglobin (Hp) concentration and the presence of udder pathogens (Strep. agalactiae, Staph. aureus, M. bovis, and Strep. uberis) in udder quarter milk samples before and after dry period. Aseptic udder quarter milk samples (n = 1,001) were collected from 133 dairy cows at dry off and at the first milking after calving from 1 large dairy herd. Bacterial DNA of Strep. agalactiae, Staph. aureus, Strep. uberis, and M. bovis in the udder quarter milk samples was identified with commercial quantitative PCR analysis Mastitis 4B (DNA Diagnostic A/S, Risskov, Denmark). Milk Hp concentration (mg/L) was measured from udder quarter milk samples. The elimination rates during the dry period for M. bovis, Staph. aureus, Strep. agalactiae, and Strep. uberis were 86.7, 93.6, 96.2, and 100.0%, respectively. The New IMI rate was 3.0% for M. bovis, 2.9% for Staph. aureus, 2.4% for Strep. agalactiae, and 3.1% for Strep. uberis. The milk Hp concentration was significantly higher in udder quarter milk samples with blood and in samples positive for Strep. agalactiae at dry off and for Staph. aureus postcalving. Elevated milk Hp concentration was not associated with the presence of M. bovis in the udder quarter milk samples. In conclusion, elimination of Staph. aureus, Strep. agalactiae, and Strep. uberis during the dry period was high; the elimination of M. bovis from infected udder quarters was lower, but probably spontaneous. Additionally, milk Hp concentration may be used as a marker for udder inflammation when combined with the bacteriological results at dry off and postpartum.

Key words: udder pathogens, dry period, elimination, haptoglobin

INTRODUCTION

Mastitis is one of the major concerns in dairy herds because it causes economic losses to the dairy industry due to lower milk yield and reduced milk quality (Hertl et al., 2014). Cow mammary glands are more susceptible to the invasion of udder pathogens at dry off and around calving. Natural defense mechanisms, such as lactoferrin, leukocytes, and keratin plug at the teat end, are present in the mammary gland, inhibiting the invasion and growth of udder pathogens during the dry period. To enhance the elimination of udder pathogens from infected udder quarters, dry cow antibiotic therapy alone or together with internal teat-sealants is used at dry off (Bradley and Green, 2004). Usually, treatment against Strep. agalactiae is effective during the dry period, but recovery from Staph. aureus is more difficult; however, some strain differences affect the elimination of Staph. aureus in dry cows (Dingwell et al., 2006). Dry cow therapy is considered ineffective in the elimination of Mycoplasma bovis from infected udder quarters (Ruegg and Erskine, 2015). Traditionally, the presence of udder pathogens before and after calving has been identified with culture-based methods, but molecular methods, such as PCR, have become more common in detecting udder pathogens from milk samples worldwide (Koskinen et al., 2010). The mammary environment during the dry period is not advantageous for udder pathogens, and acute clinical mastitis rarely occurs in dry cows (Bradley and Green, 2004). However, subclinical IMI during the dry period serves as a risk factor for clinical mastitis after calving (Bradley and Green, 2004). Mastitis-causing bacteria entering the mammary gland via the teat canal trigger a local inflammatory response and increase the level of acute phase proteins in milk (Pyörälä, 2003). Haptoglobin (Hp), one of the acute phase proteins, is mainly produced in the liver, but local production of Hp in mammary gland occurs as Hp is released from the damaged epithelial cells and neutrophils of udder...
tissues (Hiss et al., 2004). Milk Hp concentration can be used as a marker for the detection of udder inflammation (Pyörälä, 2003), as both clinical and subclinical IMI induce elevated milk Hp concentrations (Nielsen et al., 2004; Kalmus et al., 2013).

The elimination rates of Staph. aureus, Strep. agalactiae, and Strep. uberis during the dry period (Bradley et al., 2015) and the associations between these udder pathogens and milk Hp concentration (Eckersall et al., 2006; Pyörälä et al., 2011) have been previously described. No published studies have examined the elimination of M. bovis during the dry period. Additionally, associations between M. bovis and local inflammatory response in the mammary glands measured through milk Hp concentration have not been studied to our knowledge. The aim of our study was to evaluate the elimination of Strep. agalactiae, Staph. aureus, M. bovis, and Strep. uberis from infected udder quarters during the dry period using quantitative PCR (qPCR) method. The second aim of our study was to evaluate the association between milk Hp concentration and the presence of udder pathogens (Strep. agalactiae, Staph. aureus, M. bovis, and Strep. uberis) in udder quarter milk samples at dry off and postcalving.

MATERIALS AND METHODS

Study Design

Cow aseptic udder quarter milk samples were collected once at dry off and from the first milking after calving between November 2014 and May 2015 from 1 Estonian dairy herd. All the cows that were dried off and calved during that period were sampled.

All the collected udder quarter milk samples (n = 1,001) were analyzed with qPCR for the detection of bacterial DNA of Staph. aureus, Strep. agalactiae, M. bovis, and Strep. uberis. Based on the results of the qPCR analysis, the elimination rates and new infection rates were calculated. After qPCR analysis, milk Hp concentration (mg/L) was measured from all collected udder quarter milk samples to evaluate the associations between the presence of udder pathogens and milk Hp concentration.

Characteristics of the Study Herd

The milk samples were collected from 1 Estonian large loose-housed dairy herd from northeastern Estonia. Mycoplasma bovis was previously identified in bulk tank milk samples and cow composite milk samples in clinical mastitis cases in 2013. The study herd included 611 dairy cows, of which 89% were Estonian Holstein and 11% Estonian Red. Cows were milked twice per day in a 2 × 12 parallel milking parlor. The average 305-d milk yield was 9,916 kg, and the bulk milk SCC ranged between 259,000 and 358,000 cells/mL in 2014. All cows were treated with cloxacinil-based dry cow antibiotic product (Noroclox DC, 600 mg, Norbrook Laboratories Limited, Newry, UK) at dry off. The length of the dry period ranged between 37 and 94 d (median 65 d). Cow parity, DIM, and the length of the dry period were recorded from the database of Estonian Livestock Performance Recording Ltd. (Tartu, Estonia).

Collection of Udder Quarter Milk Samples

Cow udder quarter milk samples were collected at dry off and at the first milking postpartum. Before collection, the teat end was cleaned with 70% ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2 to 4 mL) were collected into sterile 10-mL plastic tubes. Milk samples were stored at −18°C and transported to the DNA Diagnostic A/S laboratory for further analysis.

qPCR Analysis of Udder Quarter Milk Samples

Bacterial DNA from M. bovis, Staph. aureus, Strep. agalactiae, and Strep. uberis was detected by commercial quantitative qPCR test kit Mastitis 4B (DNA Diagnostic A/S). The oligos of the Mastitis 4B are designed to detect DNA of Staph. aureus, Strep. agalactiae, Strep. uberis, and M. bovis. After thawing, the milk samples were vortexed and from each sample and 500 μL of milk was used for DNA extraction before PCR analysis according to the manufacturer’s instructions (DNA Diagnostic A/S, http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction_protocol_M4B_2017.11.01.pdf). The PCR mixture consisted of 15 μL of the qPCR Master Mix and 5 μL of purified DNA. The real-time PCR instrument thermal cycler Stratagene Mx3005P (Agilent Technologies Inc., Santa Clara, CA) was used for amplification. The amplification conditions were 95°C for 1 min for 1 cycle, and 95°C for 5 s and 60°C for 25 s for 40 cycles. Cycle threshold (Ct) values were reported for all samples. For all bacteria identified in the analysis, a Ct value of ≤37.0 was considered a positive result. The assay included controls for the validation of each run, including negative DNA extraction controls, internal amplification standard (positive PCR controls), and nontemplate control. The assay was validated on both bacterial strains and milk samples by the DNA Diagnostic.
Determination of Elimination Rate During the Dry Period

According to the results of PCR analysis, udder quarters were classified as positive (P) or negative (N) for detected udder pathogens both at dry off and postpartum. Based on different infectious status combinations, elimination and new IMI rates were classified and calculated for each detected udder pathogen separately. Principles of classification of elimination and new IMI rates of the mastitis pathogen during the dry period were calculated following Dufour and Dohoo (2012) as

\[
\text{Elimination} = \frac{P_{\text{dry}} \text{ and } N_{\text{pp}}}{(P_{\text{dry}} \text{ and } N_{\text{pp}}) + (P_{\text{dry}} \text{ and } P_{\text{pp}})}, \text{ and}
\]

\[
\text{New IMI} = \frac{N_{\text{dry}} \text{ and } P_{\text{pp}}}{(N_{\text{dry}} \text{ and } N_{\text{pp}}) + (N_{\text{dry}} \text{ and } P_{\text{pp}})},
\]

where \(N_{\text{dry}}\) and \(P_{\text{dry}}\) represent infectious status at dry off and \(N_{\text{pp}}\) and \(P_{\text{pp}}\) represent infectious status postpartum. If an udder quarter was positive for 1 bacterial species at dry off and for another bacterial species postcalving, the elimination rate was calculated for the bacterial species occurring at dry off and a new IMI rate for the bacterial species occurring postcalving.

Analytical Determination of Inflammatory Response in the Udder Quarter Milk Samples

Milk Hp concentrations (mg/L) were determined by a method based on the ability of Hp to bind to hemoglobin adapted to be used for milk, as described by Kalmus et al. (2013). Optical densities of the formed complex were measured at 450 nm using a spectrophotometer. Lyophilized bovine acute phase serum was used as a standard, and calibration was carried out according to the European Union concerted action on the standardization of animal acute phase proteins number QLK5-CT-1999-0153 (Skinner, 2001). The working range of the assay was 60 to 1,900 mg/L. The inter- and intra-assay coefficient of variation values for Hp analysis were <12 and <9%, respectively.

Statistical Analyses

The qPCR test results were dichotomized for each bacterium as either presence or absence of udder pathogen by using the cutoff values (≤37.0) set by the manufacturer. Cow parity was categorized into 1, 2, 3, and ≥4 lactations and DIM into ≤300 and ≥301. According to visual evaluation, the presence of blood in milk samples was dichotomized as either presence or absence (yes = 1, no = 0).

Two models were used separately for investigating associations between milk Hp concentration and udder pathogens at dry off and after calving. To achieve a normal distribution of the outcome variable, inverse square root (1/square root of Hp) transformation was used. According to the causal diagram, the presence of blood in the milk sample, cow parity, DIM, and the length of the dry period were possible confounders in these models. A mixed tobit regression model (Figure 1) was used for estimating the associations between the milk Hp concentration and the presence of udder pathogens in udder quarter milk samples at dry off, as, at dry off, 17.8% of milk sample were under the detection limit for Hp concentrations, which would violate the regression model’s assumptions. In the tobit regression, all cases above (or below) a specific threshold value were censored, although these cases remained in the analysis. A mixed linear regression model (Figure 2) was used to estimate the associations between milk Hp level and the presence of udder pathogens in udder quarter milk samples after calving. The cow was included as a random factor to both models.

In both models, interaction terms were tested for significance to determine whether the combined effect of 2 udder pathogens differed from the sum of the individual effects of the pathogens tested. \(P\)-values ≤0.05 were considered statistically significant. Assumptions of the equal variance of the outcome in all levels of predictor variables and normal distribution of the residuals were checked graphically. Stata IC 10 (StataCorp, College Station, TX) software was used for statistical analyses.

RESULTS

Identification of Mastitis Pathogens

In total, 1,001 udder quarter milk samples were collected at dry off (n = 510) and after calving (n = 491) from 133 dairy cows. At dry off, 191 (37.5%) udder quarters out of 510 were positive for 1 or more of the detected udder pathogens. The most prevalent udder pathogen was Strep. agalactiae (n = 132; 25.9%), followed by Staph. aureus (n = 63; 12.4%) and M. bovis (n = 15; 2.9%).

After calving, 57 (11.6%) of 491 udder quarter milk samples were positive for detected udder pathogens. Mycoplasma bovis and Staph. aureus were both identified in 17 (3.5%) udder quarter milk samples. Streptococcus agalactiae and Strep. uberis were identified in 14 (2.9%) and 15 (3.1%) udder quarter milk samples, respectively.
Elimination of Udder Pathogens During the Dry Period

Among detected udder pathogens, Strep. uberis had the highest elimination rate (100.0%). Elimination rates for M. bovis, Staph. aureus, and Strep. agalactiae were 86.7, 93.6, and 96.2%, respectively (Table 1).

Inflammatory Response in the Udder Quarters Before and After Dry Period

The milk Hp concentration was below the working range (≤60 mg/L) in 91 (17.8%) out of 510 udder quarter milk samples at dry off. After calving, the milk Hp concentration was below the working range in 1.8% (n = 9) of 491 udder quarter milk samples. In 10 (2%) postpartum and 4 (0.8%) dry off udder quarter milk samples with blood, the milk Hp concentration ranged between 181 and 4,096 mg/L.

The milk Hp concentration was higher in milk samples positive for Strep. agalactiae at dry off compared with milk samples negative for Strep. agalactiae (Figure 1). After calving, the milk Hp concentration was significantly higher in milk samples with blood and in milk samples positive for Staph. aureus compared with milk samples without blood and milk samples negative for Staph. aureus (Figure 2).

DISCUSSION

Elimination of Udder Pathogens During the Dry Period

The aim of our study was to evaluate the elimination of 4 udder pathogens from infected udder quarters during the dry period in cows treated with cloxacillin at dry off. Because only 1 herd was used for our study, the results are not directly comparable to other farms.
with different management practices and use of dry cow antibiotic therapy.

We identified a high elimination rate (86.7%) for *M. bovis* during the dry period. *Mycoplasma bovis* is resistant to many antibiotics, including cloxacillin (Rosenbusch et al., 2005), and *M. bovis* IMI is usually considered untreatable with dry cow therapy (Ruegg and Erskine, 2015). Hence, the elimination of *M. bovis* during the dry period is probably caused by other factors, such as altered cow immunity or unsuitable conditions for mycoplasma cells in dry mammary glands. Because of the small sample size, our results are only indicative, and further studies should investigate the elimination of *M. bovis* during the dry period in a larger study population.

Additionally, we identified a high elimination rate for *Strep. agalactiae* and *Strep. uberis* during the dry period, which is in line with previous studies (Dufour and Dohoo, 2013; Bradley et al., 2015). The elimination rate for *Staph. aureus* was also high in our study. The recovery from *Staph. aureus* IMI during the dry period is described to be more difficult compared with *Strep. agalactiae* IMI and to depend on the strain of *Staph. aureus*, with some strains of *Staph. aureus* being naturally more susceptible for elimination by antibiot-

### Table 1. Elimination and new IMI rate in udder quarter milk samples during the dry period (n = 513)

<table>
<thead>
<tr>
<th>Item</th>
<th>Elimination rate, n (%)</th>
<th>New IMI rate, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma bovis</em></td>
<td>13/15 (86.7)</td>
<td>15/498 (3.0)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>59/63 (93.6)</td>
<td>13/450 (2.9)</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>127/132 (96.2)</td>
<td>9/381 (2.4)</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>23/23 (100.0)</td>
<td>15/490 (3.1)</td>
</tr>
</tbody>
</table>

1 Number of negative udder quarter milk samples postcalving, positive at dry off/number of positive udder quarter milk samples at dry off.
2 Number of positive udder quarter milk samples postcalving, negative at dry off/number of negative udder quarter milk samples at dry off.
ics (Dingwell et al., 2006). We did not use genotyping methods for the characterization of the detected Staph. aureus, so we cannot draw any conclusions about the possible strain variation as a cause for the high elimination rate of Staph. aureus. An alternative explanation for the high elimination rate of Staph. aureus could be the parity of cows. A higher probability for elimination of Staph. aureus IMI during the dry period is described in cows with lower parity, that is with younger cows (Dingwell et al., 2006). In our study, most of the cows were in their first or second lactation, which may explain the better elimination rate of Staph. aureus during the dry period.

A high sensitivity of the PCR method to detect udder pathogens from milk samples was reported in Koskinen et al. (2010). To our knowledge, ours is the first study in which commercial qPCR analysis was used to detect mastitis pathogens in quarter milk samples at dry off and after calving. However, we collected cow udder quarter milk samples only once before and after the dry period, which may have increased the probability of false-negative and false-positive test results. As cows do not excrete a steady number of bacteria with milk all the time, the amount of bacterial DNA in negative udder quarter milk samples may have been below the qPCR detection limit, giving false-negative results. On the other hand, a sensitive qPCR detects the bacterial DNA also from nonviable bacteria (Nyman et al., 2016; Parker et al., 2018). Hence, in our study some qPCR-positive udder quarter milk samples may have been falsely positive due to bacterial DNA from bacteria already killed by the cows’ immune system. Repeated udder quarter milk sampling could have reduced the probability of false-negative and false-positive qPCR test results.

Cow udder quarter milk samples were analyzed with qPCR test kit detecting DNA of Strept. agalactiae, Staph. aureus, Strept. uberis, and M. bovis; therefore, we cannot draw any conclusions about the elimination of other udder pathogens. Pathogen-negative udder quarter milk samples in our study may have been positive for other udder pathogens, such as CNS, which are not detected by qPCR test kit Mastitis 4B. Additionally, this test kit detects udder pathogens at the species level; however, different strains of bacteria may circulate in the farm causing IMI. As we did not do any strain analysis, we cannot make any judgements about the variation in bacterial strains.

**Inflammatory Response in the Infected Udder Quarters Before and After Dry Period**

We evaluated milk Hp concentration in udder quarter milk samples at dry off and at first milking postpartum to find associations between the milk Hp level and the presence of detected udder pathogens in udder quarter milk samples. We found elevated milk Hp concentrations in milk samples positive for Strept. agalactiae at dry off and in milk samples positive for Staph. aureus after calving compared with udder quarter milk samples negative for these pathogens. Both Strept. agalactiae and Staph. aureus usually cause chronic subclinical mastitis (Keefe, 2012). Eckersall et al. (2006) found that experimentally induced subclinical Staph. aureus IMI increased milk Hp concentration. In our study, elevated milk Hp concentration in udder quarter milk samples positive for Strept. agalactiae at dry off and for Staph. aureus postpartum may indicate a present or recent subclinical IMI caused by these udder pathogens. Milk Hp may be affected by multiple udder pathogens, and the number of bacteria detected in our study was limited to 4 main gram-positive udder pathogens. Therefore, the milk Hp concentration in udder quarter milk samples may have been affected by udder pathogens not detected in the qPCR test kit used in our study. Further studies with larger sample size are needed to evaluate the association between subclinical udder inflammation and the milk Hp concentration, and the results of our study should be considered as indicative.

We did not find a significant correlation between the milk Hp concentration and the presence of M. bovis in udder quarter milk samples at dry off or after calving. To our knowledge, no published data exists about the presence of M. bovis in udder quarters and local inflammatory response in mammary gland measured via milk Hp concentration. Therefore, further studies are needed to understand the factors affecting milk Hp concentration in M. bovis-positive udder quarters.

**CONCLUSIONS**

The elimination of Strept. agalactiae, Staph. aureus and Strept. uberis during the dry period was high. The elimination of M. bovis during the dry period was lower compared with the elimination of Strept. agalactiae, Staph aureus, or Strept. uberis. Results of this study indicate that spontaneous elimination of M. bovis probably occurs during the dry period. However, further studies should investigate the elimination rate of M. bovis during the dry period in a larger population. Higher milk Hp concentrations at dry off were identified in Strept. agalactiae-positive udder quarter milk samples compared with pathogen-negative udder quarter milk samples; the same association was found for Staph. aureus postpartum. We can conclude that the milk Hp concentration can be used as an indicator of the pres-
ence of udder inflammation at dry off and after calving together with bacteriological results.

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REFERENCES


