



Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens

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ABSTRACT

Results of a commercial real-time PCR analysis for 11 mastitis pathogens from bulk tank milk (BTM) samples from all 4,258 Danish dairy herds in November 2009 to January 2010 were compared with somatic cell count (SCC) and total bacteria count (TBC) estimates in BTM. For *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*, a low real-time PCR cycle threshold (Ct) value (corresponding to high bacterial DNA quantity) was correlated with higher SCC and higher TBC. For *Staphylococcus aureus*, low Ct values were correlated only with higher SCC. For the environmental mastitis pathogens *Klebsiella* spp., *Enterococcus* spp., and *Escherichia coli*, low Ct values had a correlation with higher TBC. *Staphylococcus* spp. were found in the BTM from all herds, *Strep. uberis* in 95%, *Staph. aureus* in 91%, and *Strep. dysgalactiae* in 86%, whereas *E. coli*, *Klebsiella*, and *Strep. agalactiae* were found in 61, 13, and 7% of the herds. It is concluded that the real-time PCR used provides results that are related to the milk quality in the herds. Real-time PCR can be used in the same way as culture for monitoring BTM samples, and is especially useful for bacteria with low prevalence (e.g., *Strep. agalactiae*).

Key words: real-time polymerase chain reaction, bulk tank milk, milk quality, *Streptococcus agalactiae*

INTRODUCTION

Mastitis is the most common and costly contagious disease affecting dairy farms in the western world (Barkema et al., 2009). Bulk milk SCC (BTSCC) is routinely considered an indicator of subclinical mastitis in dairy herds. In Denmark, BTSCC and treatment frequency are the 2 main parameters for monitoring the overall mastitis situation in the country. Bacterial culture-based identification of mastitis pathogens other than *Streptococcus agalactiae* from bulk tank samples

is currently not used in Denmark. Danish veterinarians routinely use bacterial culture of quarter milk samples to improve mastitis management and to guide treatment; it remains unclear whether an annual screening program for all main mastitis pathogens would be useful. In contrast, bulk tank culture is widely accepted in many other countries as a useful tool for evaluating the quality of milk and monitoring udder-health status in a herd (Jayarao and Wolfgang, 2003; Barkema et al., 2009). Several studies have shown a correlation between mastitis pathogens in the bulk tank milk (BTM) and BTSCC and total bacteria count (TBC; Erskine et al., 1987; Keefe et al., 1997; Phuektes et al., 2003; Zadoks et al., 2004; Ryšánek et al., 2009).

Since 1963, Denmark has had a continuous surveillance program for *Strep. agalactiae*. The program involves annual *Strep. agalactiae* testing of BTM from all herds using conventional bacterial culture. Culture-positive herds are recorded in a public database, which serves as a tool for dairy farmers who are buying cattle. Although this program has been useful for measuring the overall prevalence of *Strep. agalactiae*, it has not helped to control the incidence of infections; instead, from the year 2000 onward, *Strep. agalactiae* prevalence has increased considerably from less than 2% before year 2000 to 6.1% of all Danish dairy herds in 2009 (Katholm, 2010).

Bacterial culture of milk samples on blood agar plates has for many years been the standard method for identification of mastitis pathogens. The sensitivity of culture of *Strep. agalactiae* from bulk milk samples is commonly debated. Depending on the culture media used, the inoculum volumes applied, and the specific design of different studies, the sensitivity of detecting *Strep. agalactiae* from BTM varies between 20.5 and 78% (Keefe, 1997). Because the sensitivity of a single bulk milk culture is limited, longitudinal monitoring is necessary to achieve high herd-level sensitivity of pathogen detection (Fox et al., 2005; Olde Riekerink et al., 2006).

Molecular methods have been suggested to improve the sensitivity of intramammary pathogen detection

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since the 1990s (e.g., Oliver et al., 1998; Marcos et al., 1999; Koskinen et al., 2010). In particular, PCR-based methods are increasingly being used in mastitis diagnostics. For example, Koskinen et al. (2010) compared PCR and culture using 1,000 milk samples from cows with clinical and subclinical mastitis and from healthy cows and concluded that PCR provided higher sensitivity than culture for the detection of several bacteria.

The objective of this study was to investigate the prevalence of 11 IMI pathogens as well as the β -lactamase penicillin resistance gene in BTM by using a PCR test. Further, our aim was to evaluate milk quality parameters in BTSCC and bulk tank TBC in relation to PCR results for mastitis pathogens.

MATERIALS AND METHODS

Samples and Logistics

Bulk tank milk samples from all dairy herds registered in Denmark as of December 31, 2009, were included in the study ($n = 4,258$). The average herd size and milk production in Denmark in 2009 was 121 cows and 9,022 kg of ECM/cow per year (Danish Dairy Board, 2011).

One sample was collected from each herd for bacterial culture and PCR testing between October 20, 2009, and January 6, 2010. Bulk tank milk sampling was performed during normal milk collection using a VM OVP sampling device (VM Tarm A/S, Tarm, Denmark). The collection device excluded the first 30 to 40 L of milk and thereafter sampled 1.5 mL for 40 times at regular intervals, delivering a final sample volume of 60 mL. In the last step, the device was cleaned with pressurized air. The samples were immediately stored on ice and delivered to Eurofins Steins Laboratorium A/S (Holstebro, Denmark) within 24 h. Samples for TBC and BTSCC were collected by the same procedure. According to the Danish milk quality program, TBC was measured once every second week in all herds. Bulk tank SCC was measured at each delivery (daily or every second day) in most herds and once a week for 9.4% of the herds. The samples used for TBC and BTSCC in this study were the latest ordinary samples tested. The time between sampling for PCR testing and TBC varied between 0 and 12 d, with 42% of the samples within 2 d and 83% within 6 d. Bulk tank SCC was measured on the same day as the PCR testing in 90.6% of the herds and within 4 d from the PCR testing in the rest of the herds.

Milk Quality Testing

Bulk tank SCC was measured by flow cytometry using a Fossomatic 5000 instrument (Foss, Hillerød,

Denmark). Total bacteria count was measured by flow cytometry using a Bactocount instrument (Bentley Instruments, Chaska, MN) calibrated to provide results comparable with the standard plate count. The results were recorded in the Danish Cattle Database.

Microbial Testing Procedures

The samples were tested for 11 mastitis pathogens and the staphylococcal β -lactamase gene *blaZ* (responsible for penicillin resistance in staphylococci) by using real-time PCR (PathoProof Mastitis PCR Assay, Thermo Fisher Scientific, Vantaa, Finland). The PCR test was used for direct DNA extraction and real-time PCR (i.e., without any culture procedures). The test took approximately 4 h from start to finish. A total of 350 μ L of milk was used as a starting volume for DNA extraction. The DNA extraction protocol involved an enzymatic lysis step, disrupting the cell walls of gram-positive and gram-negative bacteria, as well as spin column-based DNA purification and elution steps. Before scoring any given reaction as negative, acceptable PCR conditions were always confirmed by verifying that the cycle threshold (C_t) values and the shape of the amplification curves of the internal amplification controls were acceptable (see Koskinen et al., 2009, for further information). A negative control (distilled H₂O) was included in every real-time PCR run for confirmation that cross-contamination had not occurred in the laboratory. All additional protocol details were identical to those described elsewhere (Koskinen et al., 2009; Taponen et al., 2009).

The C_t values obtained were recorded for all samples. The C_t value is the measurement used for analyzing real-time PCR results. It represents the number of PCR cycles required to reach a set threshold fluorescence signal level. The fewer cycles it takes to obtain the threshold level, the greater the amount of template DNA in the sample. Because PCR amplification of the assay proceeds in a manner whereby DNA approximately doubles in each cycle, a C_t difference of, for example, 1 between any 2 samples corresponds to a 2 \times difference in the bacterial DNA amount in the samples.

For samples positive for *Strep. agalactiae* in culture but negative in PCR, part of the microbial 16S rRNA gene was sequenced if a pure culture could be obtained (17 out of 20 samples). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sequenced at MWG Biotech (Ebersberg, Germany). Satisfactory quality of each sequencing electropherogram was confirmed visually. The nucleotide sequences were compared with GenBank sequences using the Basic Local Alignment Search Tool. Pair-wise sequence alignments were performed using

the program DNA Star (DNASTAR Inc., Madison, WI).

Statistical Methods

Descriptive Statistics. Descriptive statistics for the distribution of Ct values were calculated for each bacterial gene. The proportion of herds with a positive reaction (Ct <40) was calculated, and the mean value, standard deviation, and distribution of the values for herds with positive results (Ct <40) were calculated.

Statistical Analyses. The correlations between Ct values for the different bacterial DNA and BTSCC and TBC were analyzed. First, a generalized additive model was made using PROC GAM in SAS version 9.3 (SAS Institute Inc., Cary, NC) to explore changes in correlation with changing Ct values. The curve fitted by the GAM model was plotted together with the raw Ct values to get an idea of the best model for a possible correlation. For all pathogens in which a correlation was seen, a linear correlation for Ct values below a certain breakpoint and a constant value above the breakpoint seemed to be the best model. The breakpoint of the curve was different for the different pathogens. The breakpoint was established based on PROC GAM, and Ct values above the breakpoint were included separately in the model by using a piecewise regression model. Models including both untransformed and log-transformed BTSCC and TBC were tested to identify the best models. The preceding BTSCC without logarithmic transformation was used as the outcome in the linear regression model. A logarithmic transformation of the preceding TBC was used as the outcome for the linear regression model. The correlations between BTSCC and TBC and the PCR reaction were determined using a linear mixed model that included all bacterial genes with a significant effect ($P = 0.05$) on the outcome variable. The pathogens of most interest (*Staphylococcus aureus*, *Strep. agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Escherichia coli*) were kept in the model even in case of nonsignificant effects.

To evaluate the effect of *Strep. agalactiae* in BTM on the risk of TBC above the milk quality thresholds of 30,000, 50,000 and 100,000 cfu/mL, data on TBC from a period of 180 d before the PCR test was analyzed. The prevalence of TBC above the limits was calculated for herds with a PCR reaction at ≤ 32 or > 32 for *Strep. agalactiae*, and the difference was tested by a *t*-test using PROC TTEST in SAS version 9.3 (SAS Institute Inc.). The Ct value of 32 was chosen based on the breakpoint for analysis of the effect of PCR reaction for *Strep. agalactiae* on TBC.

RESULTS

Real-Time PCR Analysis

Descriptive statistics of the Ct values for all 12 PCR targets are presented in Table 1. All the species detected by the PCR assay were commonly found in the BTM of Danish dairy herds, except *Strep. agalactiae* (found in 7% of the samples), *Klebsiella* (13%), and *Serratia marcescens* (2%). The only species found in all herds were *Staph. spp.* The major contagious mastitis pathogens and *Strep. uberis* had low median values (29.8 to 31.6) for positive herds compared with bacteria considered common environmental bacteria (*E. coli*, *Enterococcus spp.*, *Klebsiella spp.*, *S. marcescens*; median 33.7 to 37.8).

Ct Values and BTM Quality

The breakpoints for the correlation between Ct value and BTSCC were decided as 28 for *E. coli*, 32 for *Strep. agalactiae* and *Strep. dysgalactiae*, 33.5 for *Staph. aureus*, and 34.5 for *Strep. uberis*. Selected graphs show the correlation for *Strep. agalactiae*, *Strep. dysgalactiae*, *Staph. aureus*, *Strep. uberis*, and *E. coli* in Figure 1. The effect of *E. coli* on BTSCC was not significant but was kept in the model to show its lack of importance. Removing *E. coli* from the model had a marginal effect on the estimates for the other bacteria in the model. The baseline BTSCC was estimated to 184,562 cells/mL and the BTSCC increased with a decrease in the Ct value, with 10,389, 9,074, 4,178, and 3,707 cells/mL per 1-unit change in Ct below the breakpoints for *Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, and *Strep. agalactiae*, respectively.

The breakpoints for the correlation between Ct value and TBC were decided as 30.5 for *Staph. aureus*, 32 for *Strep. agalactiae*, 32 for *Strep. dysgalactiae*, and 35 for *Strep. uberis*. For *E. coli*, *Enterococcus spp.*, and *Klebsiella spp.*, the breakpoint was 40. Selected graphs showing the correlations are presented for *Strep. agalactiae*, *Strep. dysgalactiae*, *Staph. aureus*, *Strep. uberis*, *Enterococcus spp.*, *E. coli*, and *Klebsiella spp.* in Figure 2. The effect of *Staph. aureus* on TBC was not significant but was kept in the model to show its lack of importance. The baseline TBC was estimated to 7,508 cfu/mL, and the TBC increased with decreasing Ct values for the bacteria presented. The prevalence values of TBC above the Danish milk quality threshold of 30,000, 50,000, and 100,000 cfu/mL were higher in herds with a *Strep. agalactiae* Ct value at or below 32 (Table 2).

Table 1. Results of a real-time PCR test¹ of bulk tank milk samples in 4,258 Danish dairy herds sampled from October 2009 to January 2010

| PCR target | Distribution of Ct values for samples with Ct <40 ² | | | | | | | | |
|---|--|------|-----|--------------|-------------|-------------|--------|-------------|-------------|
| | % Ct <40 | Mean | SD | Lowest value | Fractile 10 | Fractile 25 | Median | Fractile 75 | Fractile 90 |
| <i>Staphylococcus aureus</i> | 91 | 32.5 | 2.9 | 19.5 | 28.9 | 30.5 | 32.4 | 34.4 | 36.3 |
| <i>Staphylococcus</i> spp. | 100 | 29.8 | 2.1 | 17.7 | 27.3 | 28.6 | 29.8 | 31.0 | 32.1 |
| β -Lactamase | 78 | 34.8 | 2.7 | 22.2 | 31.5 | 33.1 | 34.8 | 36.6 | 38.4 |
| <i>Streptococcus agalactiae</i> | 7 | 31.5 | 4.8 | 17.3 | 25.7 | 28.5 | 31.5 | 35.1 | 37.9 |
| <i>Streptococcus dysgalactiae</i> | 86 | 31.6 | 3.2 | 15.9 | 27.7 | 29.6 | 31.6 | 33.6 | 35.6 |
| <i>Streptococcus uberis</i> | 95 | 30.3 | 3.4 | 13.9 | 26.0 | 28.1 | 30.3 | 32.4 | 34.5 |
| <i>Corynebacterium bovis</i> | 90 | 33.7 | 1.7 | 24.5 | 31.9 | 32.6 | 33.5 | 34.5 | 35.8 |
| <i>Enterococcus</i> spp. | 78 | 33.6 | 2.8 | 20.8 | 30.0 | 31.9 | 33.7 | 35.6 | 37.3 |
| <i>Escherichia coli</i> | 61 | 35.2 | 3.5 | 17.6 | 30.4 | 33.2 | 35.8 | 38.1 | 39.3 |
| <i>Klebsiella</i> spp. | 13 | 35.8 | 3.5 | 18.9 | 31.3 | 33.7 | 36.5 | 38.6 | 39.6 |
| <i>Serratia marcescens</i> | 2 | 37.0 | 3.0 | 25.4 | 33.8 | 36.4 | 37.8 | 38.9 | 39.4 |
| <i>A. pyogenes</i> / <i>P. indolicus</i> ³ | 63 | 35.2 | 2.9 | 18.5 | 31.8 | 33.9 | 35.7 | 37.2 | 38.5 |

¹PathoProof Mastitis PCR Assay (Thermo Fisher Scientific, Vantaa, Finland).

²Ct = 40 is regarded as a negative test. Ct = cycle threshold.

³*A. pyogenes*/*P. indolicus*: *Arcanobacterium pyogenes*/*Peptococcus indolicus*.

DISCUSSION

Prevalence of Pathogens

Monitoring of major mastitis pathogens in BTM at the population level has been described in only a few studies. Ryšánek et al. (2009) sampled 268 randomly selected herds. The prevalence by culture was 19% for *Enterococcus* spp. (*E. faecium* and *E. faecalis*), 14% for *Strep. uberis*, 13% for *Strep. dysgalactiae*, 12% for *Staph. aureus*, 7% for *E. coli*, and 3% for *Strep. agalactiae*.

In a study in all 258 herds on Prince Edward Island, Canada, 3 consecutive BTM samples were cultured at weekly intervals. Cumulative prevalence values of *Staph. aureus* and *Strep. agalactiae* were 74 and 1.6%, respectively (Olde Riekerink et al., 2006). Zadoks et al. (2004) cultured BTM samples from 48 New York State dairy herds and found 31% positive for *Strep. agalactiae* and 81% positive for *Strep. uberis*. Phuektes et al. (2003) tested BTM from 42 herds in Australia on 5 occasions at approximately 10-d intervals with a multiplex PCR. The herds were selected based on low,

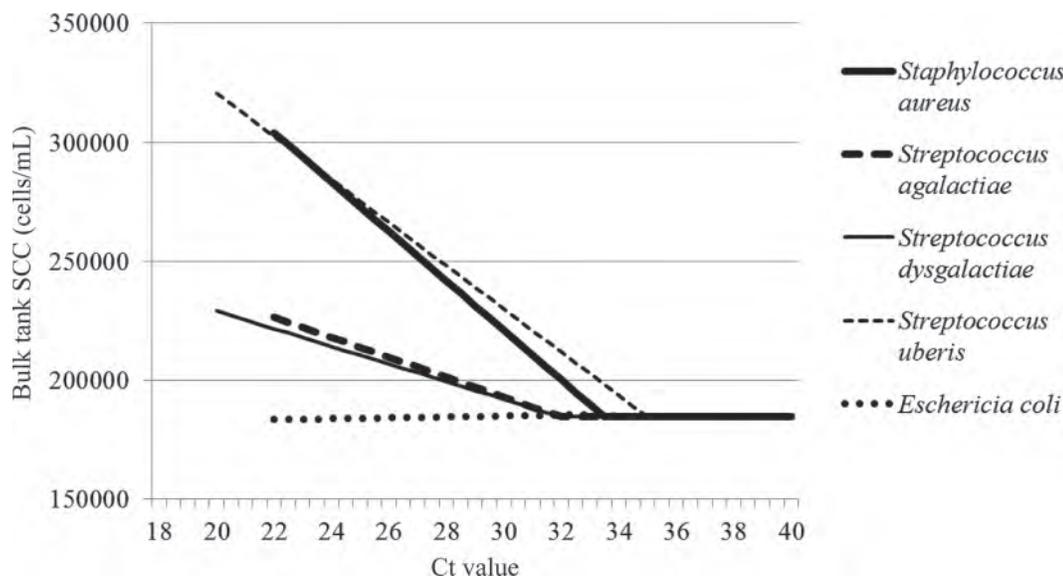


Figure 1. Correlation between cycle threshold (Ct) values of real-time PCR (PathoProof Mastitis PCR Assay, Thermo Fisher Scientific, Vantaa, Finland) for different bacteria and bulk tank SCC estimated from a linear mixed model. The effect of *Escherichia coli* was not different from zero.

Table 2. Herd prevalence (%; 95% CI in parentheses) of total bacteria count in bulk tank milk above the limits of 30,000, 50,000, and 100,000 cfu/mL the last 180 d before bulk tank milk PCR testing in 4,258 Danish dairy herds with a cycle threshold (Ct) value above or below 32 for *Streptococcus agalactiae*

| Total bacteria count (cfu/mL) | Ct value for <i>Strep. agalactiae</i> | |
|-------------------------------|---------------------------------------|----------------------------|
| | ≤32 | >32 |
| >30,000 | 12.1 ^a (9.6–14.6) | 7.3 ^b (6.8–7.7) |
| >50,000 | 5.5 ^a (4.2–6.9) | 3.3 ^b (3.1–3.6) |
| 100,000 | 2.2 ^a (1.4–3.0) | 1.5 ^b (1.3–1.6) |

^{a,b}Different letters within a row indicate significant differences ($P = 0.05$).

medium, or high BTSCC. They found *Strep. uberis* in at least 1 of the 5 samples in 83%, *Strep. dysgalactiae* in 55%, *Staph. aureus* in 33%, and *Strep. agalactiae* in 26% of the herds. If *Strep. agalactiae* was detected by PCR, it was constantly detected in repeated samples collected from the herd. Compared with the referenced studies, the prevalence values of *Staph. aureus* and *Strep. dysgalactiae* were high in the Danish herds. The higher prevalence of these udder-specific bacteria might be influenced by the limited use of dry cow treatments in Danish herds because of legislation prohibiting the use of dry cow antibiotics without a microbiological diagnosis or clinical mastitis (Danish Veterinary and Food Administration, 2010). The prevalence of *Strep. agalactiae* was low compared with the results from New

York state and Australia but high compared with the Canadian study. In Denmark, *Strep. agalactiae* has been regulated by a mandatory annual surveillance with registration of infected herds and restrictions against the sale of livestock, but has been increasing from approximately 2% in 2000 to the current level of 6.1% in 2009 (Katholm, 2010). In addition, other Nordic countries have reported similar problems. In Sweden, 4.9% of BTM samples from 465 herds with automatic milking systems were found to be positive for *Strep. agalactiae*; in Norway, 3.3% of BTM samples from 936 larger herds (more than 35 cows) were positive for *Strep. agalactiae*; and on Faroe Island, 7 herds (23%) were positive for *Strep. agalactiae* in a test of BTM from all 30 dairy herds in 2010. All 3 investigations were performed using the PathoProof PCR test (Katholm, 2010).

The low median Ct value for major contagious mastitis pathogens (*Strep. agalactiae*, *Strep. dysgalactiae*, *Staph. aureus*) compared with the median values of *E. coli*, *Enterococcus* spp., and *Klebsiella* spp. might be a result of subclinically infected cows shedding bacteria in high numbers, which, in herds with a high prevalence of the bacteria, results in very high numbers of bacteria in the BTM. These environmental bacteria would be shed for only a short time by cows with clinical mastitis, and most bacteria in the milk and their presence in BTM are suggestive of problems related to stall management, udder hygiene, and milking practices, resulting in con-

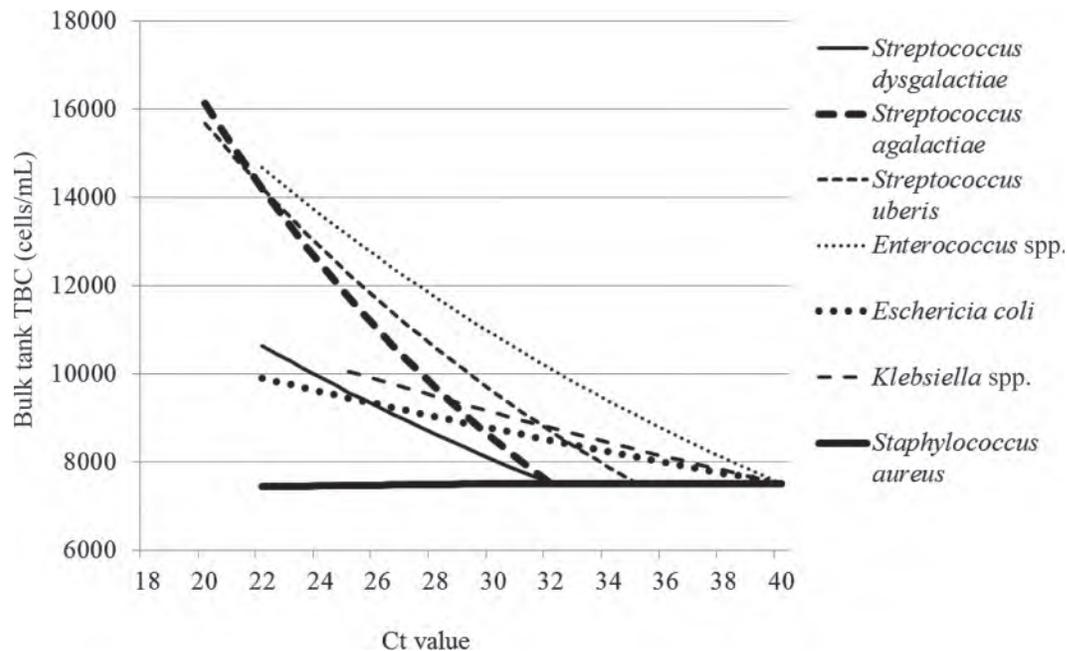


Figure 2. Correlation between cycle threshold (Ct) values of real-time PCR (PathoProof Mastitis PCR Assay, Thermo Fisher Scientific, Vantaa, Finland) for different bacteria and bulk tank total bacterial counts (TBC) estimated from a linear mixed model. The effect of *Staphylococcus aureus* was not different from zero.

tamination of the milk (Jayarao and Wolfgang, 2003; Hogan and Smith, 2012).

PCR as an Indicator of Milk Quality

Analysis of the association between BTSCC and TBC and the Ct values (Figures 1 and 2) estimates the influence of the different bacteria on milk quality. The difference regarding the Ct value for different bacteria might be a result of *E. coli* and *Enterococcus* spp. acting as indicator bacteria, representing general hygiene problems in the herds resulting in contamination of the milk in the bulk tank, with many different bacteria all accounting for the TBC, whereas the effect of the more udder-specific bacteria, such as *Strep. uberis* and *Strep. agalactiae* on TBC might be the result of the specific bacteria being shed from infected quarters in very high numbers. *Streptococcus uberis* had a high threshold value of 35, which might be a result of the bacteria being shed from infected cows but also being an environmental species present in high numbers in herds with poor hygiene. We found no significant correlation between *Staph. aureus* Ct value and TBC. *Staphylococcus aureus* is not thought to be a frequent contributor to TBC (Gonzalez et al., 1986; Jeffrey and Wilson, 1987; Fenlon et al., 1995).

In general, the environmental mastitis bacteria, including *Enterococcus* spp., *E. coli*, and *Klebsiella*, had high median Ct values in positive herds compared with the contagious mastitis pathogens, indicating that they were found in lower numbers. In cases with high *E. coli* Ct values, the BTSCC was generally not affected. This is not surprising because *E. coli* udder infections normally are of short duration and often the milk will be visibly changed and subsequently discarded and not entering the bulk tank. *Enterococcus* spp. are rarely found to be the primary cause of mastitis and are not expected to cause prolonged udder infection with elevated cell counts.

For the other major mastitis pathogens, the correlation between Ct values and BTSCC was strongest for *Staph. aureus* and *Strep. uberis*. These 2 species are the main causes of high SCC, based on our results. This result is in agreement with other studies with culture of milk samples from both clinical cases and cross-sectional sampling (Barkema et al., 1998; Jayarao et al., 2004; Ryšánek et al., 2009). The Ct values for *Strep. dysgalactiae* were at the same level as those of *Staph. aureus*, but the effect on the BTSCC was much less (approximately 50%). *Streptococcus agalactiae* also had a significant effect on the BTSCC but was found in only 7% of the herds, which is in accordance with other studies (Erskine et al., 1987; Keefe et al., 1997; Phuektes et al., 2003).

The Ct values for *Corynebacterium bovis* (Table 1) were characterized by a very low standard deviation and a median at 34. Although it could be expected to be a potential indicator for low udder hygiene, the low standard deviation makes it of little value for characterization of the herds.

BTM Bacteria and Milk Quality

Bacteriological testing of raw BTM is used as an indicator of udder health, milk harvest hygiene, and storage conditions on the farm. Our TBC results might have been affected by the fact that TBC was not necessarily measured on the same day as the samples for PCR. The difference in sampling times might result in an underestimation of the effect of the different bacteria. The effect of the udder-specific mastitis pathogens, such as *Strep. agalactiae*, on TBC is probably related to the excretion of bacteria in high numbers from both acute and chronic cases. *Streptococcus agalactiae* and *Strep. uberis* have been found to be shed in very high numbers (up to 10^9 bacteria/mL from infected quarters; Guterbock and Blackmer 1984; Schukken et al., 2011).

The higher prevalence of TBC above 30,000, 50,000, and 100,000 cfu/mL in herds with a *Strep. agalactiae* Ct value ≤ 32 (Table 2) is in accordance with the correlation between Ct values and TBC (Figure 2). Accordingly, Keefe et al. (1997) found that herds infected with *Strep. agalactiae* were 5.48 times more likely to be penalized for a high standard plate count. Our results are also in agreement with those of Jeffrey and Wilson (1987), who found that 28.1% of all failed TBC tests were caused by more than 90% of mastitis bacteria present in the milk. *Streptococcus uberis* accounted for 50% of the cases and *Strep. agalactiae* accounted for 33% of the cases.

For *Strep. agalactiae*, a correlation between the proportion of shedding cows and TBC has previously been shown to exist in many but not all herds, and discrepancies between the proportion of shedding cows and counts in BTM was attributed to variation in levels of shedding among cows (Gonzalez et al., 1986; Farnsworth, 1993). Zadoks et al. (2004) concluded that infected cows were the source of *Strep. uberis* in raw BTM because the bacteria in the bulk tank was of the same strain as the bacteria in infected cows.

Repeated sampling of BTM is needed to determine a true prevalence estimate of contagious mastitis pathogens (Godkin and Leslie, 1993; Ruegg and Reinemann, 2002). Therefore, we expect that repeated sampling of BTM will provide yet more precise estimations of the role of the different pathogens. This will be evaluated in future investigations.

CONCLUSIONS

Real-time PCR for mastitis bacteria in BTM can be used in the same way as culture for monitoring BTM samples, especially for bacteria with low prevalence (e.g., *Strep. agalactiae*).

We have presented baseline figures for 11 mastitis pathogens and the β -lactamase gene in BTM samples from all Danish dairy herds in 2009. High levels of mastitis bacteria are related to decreased milk quality. We found that high PCR-based quantities (low Ct values) of *Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, and *Strep. agalactiae* correlated with high BTSCC and that low PCR-based quantities (high Ct values) of *Strep. agalactiae*, *Strep. uberis*, *Enterococcus* spp., *Strep. dysgalactiae*, *E. coli*, and *Klebsiella* spp. correlated with high TBC.

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