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SHORT COMMUNICATION

Evaluation of a new qPCR test to identify the organisms causing high total bacterial count in bulk tank milk

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Abstract

Milk quality in bulk tank milk (BTM) is measured by flow cytometry technology as total bacterial count (TBC) and somatic cell count (SCC). To investigate SCC problems, culture or PCR can be used to identify mastitis causing bacteria, e.g., Mastit 4, a commercially available qPCR test. TBC in BTM can be investigated further using culture-based methods such as standard plate count, laboratory pasteurization count, coliform count, and spore counts. To our knowledge, no qPCR addressing the bacteria involved in TBC has been commercially introduced. The aim of this study is to evaluate a recently introduced 3-h qPCR test, TBC 4. The TBC 4 qPCR detects four target groups, *Pseudomonas*, *Streptococci*, *Enterobacteriacea*/*Enterococcus*, and *Bacillus*/*Clostridia*. These target groups relates to problems on the farm such as cooling, mastitis, environment, and silage. We will continue with new research to compare the TBC 4 qPCR test with traditional culture. For this study, BTM samples from different TBC intervals were selected based on BactoCount results found at routine payment investigation at Eurofins laboratory (Vejen, Denmark). These samples were analyzed using TBC 4 qPCR assay within 24 h. In total 346 BTM samples were divided into 6 different intervals of colony forming units (CFU). For all four targets in each of the different intervals of CFU, the percent of positive samples, the average C_t-value, the percent of positive samples with C_t<30 and C_t<25 were calculated. For *Pseudomonas*, *Streptococci*, and *Enterobacteriacea*/*Enterococcus* the number of positive samples with lower C_t-values (high bacteria content) correlated with the CFU mL^{-1}. We found *Enterobacteriacea*/*Enterococcus*, *Pseudomonas*, and *Streptococci* in high number of bacteria (C_t<25) in 25, 19 and 56% of samples with CFU mL^{-1} between 50 001–100 000 and 53, 44, and 39% in samples with CFU mL^{-1}>100 000. The TBC 4 qPCR test showed to be a strong and fast tool for farmers, advisors and service technicians to address problems with high TBC and ensuring the delivery of good quality milk to the dairy.

Keywords: TBC, bulk tank milk, qPCR, milk quality

1. Introduction

Milk quality in bulk tank milk (BTM) is measured by flow cytometry technology as total bacterial count (TBC) and somatic cell count (SCC). There has been a long tradition for using cultivation of BTM samples to identify different bacteria causing high SCC in the milk. Also qPCR tests, e.g., Mastit

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4, a commercially available qPCR test (DNA Diagnostic, Risskov, Denmark), can be used to detect mastitis bacteria in BTM (Rattenborg *et al*. 2015).

Tests for milk quality and bacteria in BTM includes standard plate count (SPC), coliform count (CC), and laboratory pasteurization count (LPC) (Guterbock and Blackmer 1984; Murphy 1997). Estimation of the type and number of bacteria in BTM is valuable in understanding and troubleshooting issues related to udder health, milk harvest hygiene, cleaning practices, and milk storage conditions (Elmoslemany *et al*. 2009). They investigated risk factors for bacteriological quality of bulk tank milk, highlighting the importance of udder hygiene and milking system washing factors on hygienic quality of bulk tank milk. Milking machine wash failures is strongly associated with in-line CC, which suggests that proper and consistent washes play a fundamental role in minimizing BTM contamination with coliforms (Pantoja *et al*. 2011). Increases in SPC (Costello *et al*. 2003) and slightly higher CC (Pantoja *et al*. 2011) were found after transfer of raw milk from farm tanks to dairy processor bulk tanks. Environmental contamination is an important factor for the bacterial content of milk. Vacheyrou *et al*. (2011) investigated the bacterial content in air, dust, hay, and cow teat surface and found that milk contamination by the stable environment was considerable, although it was lower in farms with a milking parlor compared to tie stalls. The study of Lucali *et al*. (2015) underlined the correlation between forage quality, dairy farm management practices and the presence of milk and cheese anaerobic spore-forming bacteria.

It is well known that *Streptococci* from mastitis cows can cause high TBC. *Streptococcus agalactiae* and *Streptococcus uberis* have been found to be shed in very high numbers (up to 10^9 bacteria mL^{-1} from infected quarters (Guterbock and Blackmer 1984; Schukken *et al*. 2011). Zadoks *et al*. (2004) found that *Streptococci*, *Staphylococci*, and Gram-negative bacteria accounted for 69, 3, and 3% of TBC variability, in 48 BTM samples from New York State dairy farms. Keefe *et al*. (1997) found that herds infected with *Strep*. *agalactiae* were 5.48 times more likely to be penalized for a high SPC. Also Gillespie *et al*. (2012) found strong correlations between SPC and *Streptococcus* spp. counts (0.72).

Detection of bacterial DNA can be used for analyses of bacterial content in BTM. Katholm *et al*. (2012) tested Danish BTM samples with qPCR and found the highest correlation to TBC for *Enterococcus*, *Strep*. *uberis* and *Strep*. *agalactiae* of the bacteria investigated. Analysis with community 16S rRNA gene sequence was used by Kable *et al*. (2016) to identify bacteria in raw bovine milk samples from tanker trucks arriving to two dairy processors in California, USA. They found the highest total cell numbers and the highest proportions being those of *Actinobacteria*. Even with this complexity, a core microbiota was present, consisting of 29 taxonomic groups and high proportions of *Streptococcus* and *Staphylococcus* and unidentified members of *Clostridiales*. To our knowledge, thus far no qPCR addressing the bacteria involved in TBC has been commercially introduced. The aim of this study is to evaluate a recently introduced 3-h qPCR test, TBC 4 (DNA Diagnostic, Denmark). We will continue to compare the TBC 4 qPCR test with traditional culture. The TBC 4 qPCR gives a C_t-value for four targets, *Pseudomonas*, *Streptococci*, *Enterobacteriacea*/*Enterococcus*, and *Bacillus*/*Clostridia*. These four targets correlates to the problems on the farm related to cooling, mastitis, environment, or silage. We were not able to compare the TBC 4 qPCR to culture in this trial but further trials will evaluate this.

2. Material and methods

In the period between 7th March and 5th April, 2017, BTM samples obtained from Eurofins laboratory (Vejen, Denmark) were measured for TBC by routine flow cytrometry with BactoCount IBC (Bentley instruments, Inc., Chaska, USA). For this study, we selected 346 milk samples from different TBC intervals for qPCR test with TBC 4. The samples were selected among all Danish dairy herds. The general descriptive data for these Danish herds are 172 cows/herd, yield is 10 008 kg per cow, almost all cows are feed total mixed ratio, average geometric mean BTM SCC is 205 800 cells mL^{-1} and geometric mean TBC is 7 690 bacterial count mL^{-1} (Danish Agriculture and Food Council 2016). Mastitis treatments per cow year was 0.33 in yield control herds with *Strep*. *uberis* and *Staphylococcus aureus* as the dominant pathogens.

After the result from the flow cytometry TBC test was obtained, the samples were immediately transported on ice to the laboratory of DNA Diagnostic A/S, Risskov, Denmark and tested by the TBC 4 qPCR test within 24 h.

3. Results

The results from the TBC 4 test of the 346 BTM samples in different groups of CFU mL^{-1} is shown in Table 1.

In total 158 (46%) samples were positive for *Pseudomonas*, 157 (45%) for *Streptococci*, 128 (37%) for *Enterobacteriacea*/*Enterococcus,* and 122 (35%) for *Bacillus*/*Clostridia*.

In each of the different intervals of TBC, the percent of positive samples, average C_t-value of positive samples, percent samples with C_t<30 and percent samples with C_t<25

were calculated for all four targets of the test (Fig. 1).

The *Pseudomonas*, *Streptococci* and the *Enterobacteriacea*/*Enterococcus* target showed increasing percent positive samples with higher CFU and also reduced C_t-value at higher CFU, indicating more of these bacteria is present at higher CFU. For *Bacillus*/*Clostridia*, the increase in positive samples stopped at 30 000 CFU mL^{-1} and the average C_t-value were above 30 in all groups of CFU (Fig. 1-A and B). The percent positive samples with C_t-value below 30 and 25 is shown in Fig. 1-C and D. As it can be seen, we did not find many *Bacillus*/*Clostridia*-positive samples with really low C_t-values. For the *Streptococci*, they have the highest percent of samples with low C_t-values in the samples up to 100 000 CFU mL^{-1}, whereas both the *Pseudomonas* and the *Enterobacteriacea*/*Enterococcus* target have the highest percent of samples with low Ct-values in the samples above 100 000 CFU mL^{-1}.

Table 1 Number of bulk tank milk samples tested in each group of CFU mL^{-1}

CFU mL^{-1}	N
≤5000	53
5001–15000	67
15001–30000	73
30001–50000	65
50001–100000	52
>100000	36
Total	346

4. Discussion

The new qPCR test TBC 4 enables the user to classify high TBC in BTM to four different groups of problems related to cooling, mastitis, environment or silage. Not all problems with high TBC are solved by optimizing cooling and the washing procedures, as we found 46% of samples positive for *Pseudomonas* and 37% for *Enterobacteriacea*/*Enterococcus*.

Of the four targets investigated by the TBC 4 qPCR test, *Pseudomonas*, *Streptococci* and *Enterobacteriacea*/*Enterococcus* seems to have the highest influence on the CFU in BTM collected during March and April, 2017 in Denmark. This is seen in the Fig. 1-B where the low C_t-values for these targets in samples with CFU mL^{-1}>30 000 indicates higher number of bacteria. We found

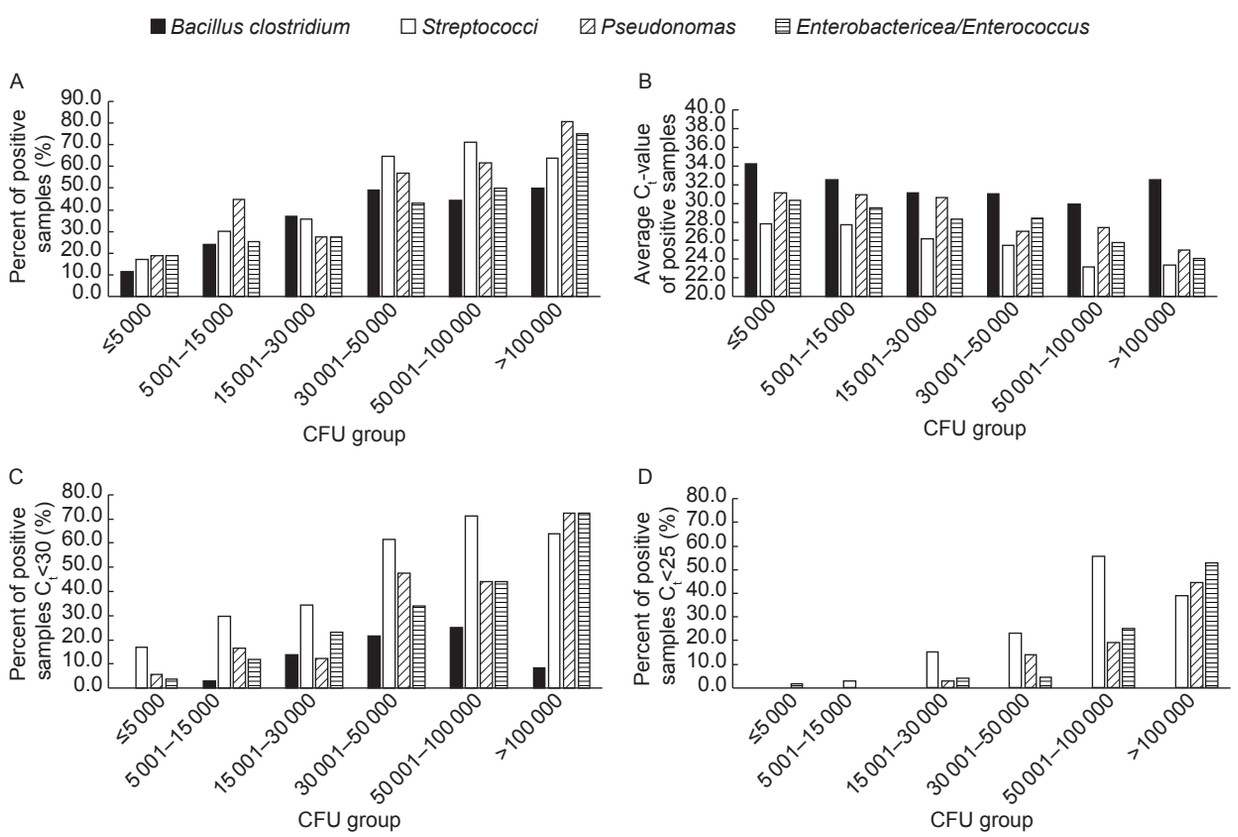

Fig. 1 A, percent of positive samples. B, average C_t-value for positive samples. C, percent C_t-value under 30. D, percent C_t-value under 25 for the different groups of colony forming units (CFU) for each of the four different targets in the qPCR test the TBC 4 (DNA Diagnostic, Denmark).

Enterobacteriacea/*Enterococcus*, *Pseudomonas*, and *Streptococci* in high number of bacteria (C_t<25, Fig. 1-D) in 25, 19 and 56% of samples with CFU mL^{-1} between 50 001–100 000 and 53, 44, and 39% in samples with CFU mL^{-1}>100 000, respectively. Holm *et al*. (2004) found, in Danish BTM samples with >30 000 CFU mL^{-1}, microorganisms primarily associated with poor hygiene dominated the microflora in 64% of the samples; bacteria also related to poor hygiene, but in addition associated with growth at low temperatures (psychrotrophic bacteria) dominated the microflora in 28% of the samples; and bacteria mainly associated with mastitis dominated the microflora in 8% of the samples. Their findings for microorganisms, primarily associated with poor hygiene and psychrotropic bacteria, corresponds with our findings for *Enterobacteriacea*/*Enterococcus* and *Pseudomonas*, whereas our data indicates much more problems related to mastitis bacteria. In contrary to the data from Holm *et al*. (2004), our test do not detect *Staphylococci* but the mastitis primer detects *Streptococci*. On the other hand, the *Streptococci* primer can also detect *Streptococci* not so often related to mastitis as e.g., *Strep*. *bovis*.

Our findings, that *Streptococci* is an important factor in high TBC, is in accordance with the findings of Keefe *et al*. (1997). Also Gillespie *et al*. (2012) and Hayes *et al*. (2001) found a strong correlation between SPC and *Streptococcus* spp. counts. Katholm *et al*. (2012) found the best correlation between TBC in bulk tank milk and C_t-values from real-time PCR assays specific for *Enterococcus*, *Strep*. *uberis* and *Strep*. *agalactiae*, less correlation to C_t-values for *Strep*. *dysgalactiae*, *Escherichia coli* and *Klebsiella*, and no correlation to *Staph*. *aureus*. These findings is in agreement with Zadoks *et al*. (2004), who found that *Streptococci*, *Staphylococci*, and Gram-negative bacteria account for 69, 3, and 3% of total bacterial count, respectively. Our findings, that the *Enterobacteriacea*/*Enterococcus* is an important finding in milk samples with high CFU is in accordance with the results from Pyz-Lukasik *et al*. (2015), who tested the microbiological quality of milk sold directly from producers to consumers in Poland. They found *Enterobacteriaceae* ranging from 6.4×10^1 to 1.7×10^6 CFU mL^{-1}.

The new TBC 4 qPCR test proved to be useful in indicating the major causes of high TBC in Danish BTM samples. We expect the test to be a strong and fast tool for farmers, advisors and service technicians to address problems with high TBC and ensuring the delivery of good quality milk to the dairy.

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References

Costello M, Rhee M S, Bates M P, Clark S, Luedecke O, Kang D H. 2003. Eleven-year trends of microbiological quality in bulk tank milk. *Food Protect Trends*, **23**, 393–400.

Danish Agriculture and Food Council. 2017. Danish Dairy Statistics. 2016. [2017-4-21]. http://www.lf.dk/tal-og-analyser/statistik/mejeri/mejeristatistik/mejeristatistik-2016

Elmoslemany A M, Keefe G P, Dohoo I R, Jayarao B M. 2009. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1: Overall risk factors. *Journal of Dairy Science*, **92**, 2634–2643.

Gillespie B E, Lewis M J, Boonyayatra S, Maxwell M L, Saxton A, Oliver S P, Almeida R A. 2012. Short communication: Evaluation of bulk tank milk microbiological quality of nine dairy farms in Tennessee. *Journal of Dairy Science*, **95**, 4275–4279.

Guterbock M, Blackmer P E. 1984. Veterinary interpretation of bulk-tank milk. *Veterinary Clinnics of North America: Large Animal Practice*, **6**, 257–268.

Hayes M C, Ralyea R D, Murphy S C, Carey N R, Scarlett J M, Boor K J. 2001. Identification and characterization of elevated microbial counts in bulk tank raw milk. *Journal of Dairy Science*, **84**, 292–298.

Holm C, Jepsen L, Larsen M, Jespersen L. 2004. Predominant microflora of downgraded danish bulk tank milk. *Journal of Dairy Science*, **87**, 1151–1157.

Kable M E, Srisengfa Y, Laird M, Zaragoza J, McLeod J, Heidenreich J, Marco M L. 2016. The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. *Mbio*, **7**, e00836-16.

Katholm J, Bennedsgaard T W, KoskinenM T, Rattenborg E. 2012. Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens. *Journal of Dairy Science*, **95**, 5702–5708.

Keefe G P, Dohoo I R, Spangler E. 1997. Herd prevalence and incidence of *Streptococcus agalactiae* in the dairy industry of prince edward island. *Journal of Dairy Science*, **80**, 464–470.

Murphy S C. 1997. Raw milk bacteria tests: Standard plate count, preliminary incubation count, lab, pasteurization count and coliform count. What do they mean for your farm? In: *National Mastitis Council*. Reg. Mtg. Proceedings, National Mastitis Council, Syracuse, NY, Madison, WI. pp. 34–41.

Pantoja J C F, Reinemann D J, Ruegg P L. 2011. Factors associated with coliform count in unpasteurized bulk milk. *Journal of Dairy Science*, **94**, 2680–2691.

Pyz-Lukasik R, Paszkiewicz W, Tatara M R, Brodzki P, Belkot

Z. 2015. Microbiological quality of milk sold directly from producers to consumers. *Journal of Dairy Science*, **98**, 4294–4301.

Rattenborg E, Paulrud C O, Jensen S K S, Katholm J. 2015. Bulk tank milk surveillance for contagious mastitis pathogens: Comparison of two commercial real-time PCR test kits. In: *Proceedings of the 54th Annual Meeting of the National Mastitis Council*. National Mastitis Council In., Madison, WI. pp. 215–216.

Schukken Y H, Gunther J, Fitzpatrick J, Fontaine M C, Goetze L, Holst O, Leigh J, Petzl W, Schuberth H J, Sipka A, Smith D G E, Quesnell R, Watts L, Yancey R, Zerbe H, Gurjar A, Zadoks R N, Seyfert H M, 2011. Pfizer mastitis research consortium. Host-response patterns of intramammary infections in dairy cows. *Veterinary Immunology and Immunopathology*, **144**, 270–289.

Vacheyrou M, Normand A C, Guyot P, Cassagne C, Piarroux R, Bouton Y. 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen *French farms. International Journal of Food Microbiology*, **146**, 253–262.

Zadoks R N, Gonzàlez R N, Boor K J, Schukken Y H. 2004. Mastitis-causing *Streptococci* are important contributors to bacterial counts in raw bulk tank milk. *Journal of Food Protection*, **67**, 2644–2650.

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