Evaluation of a new qPCR test to specify reasons behind 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 three-hour 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 TBC4 qPCR assay within 24 hours.

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 average Ct-value, percent positive samples with Ct<30 and Ct<25 were calculated.

For *Pseudomonas, Streptococci*, and *Enterobacteriacea/Enterococcus* the number of positive samples with lower Ct-values (high bacteria content) correlated with the CFU/mL. We found *Enterobacteriacea/Enterococcus*, *Pseudomonas*, and *Streptococci* in high number of bacteria (Ct <25 figure d) in 25%, 19% and 56% of samples with CFU/mL between 50,001-100,000 and 53%, 44%, and 39% in samples with CFU/mL>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, btm, quality, qpcr.

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 4 a commercially available qPCR test (DNA Diagnostic, 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) (Murphy 1997).

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). 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. 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 Your State. Keefe *et al*. (1997) found that herds infected with Strep. agalactiae were 5.48 times more likely to be penalized for a high SPC.

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.

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 three-hour qPCR test, TBC 4 (DNA Diagnostic, Risskov, Denmark). The TBC 4 qPCR gives a Ct-value for four targets, *Pseudomonas, Streptococci, Enterobacteriacea/ Enterococcus*, and *Bacillus/Clostridia*. These four targets correlates to problems on the farm related to cooling, mastitis, environment, or silage.

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. 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.

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 hours.

Results and discussion

The results from the TBC 4 test of the 346 BTM samples in different groups of CFU/mL 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 Ct-value of positive samples, percent samples with Ct. < 30 and percent samples with Ct. < 25 were calculated for all four targets of the test (Figure a,b,c, and d).

Table 1. Number of bulk tank milk samples tested in each group of CFU/mL.

CFU / mL	N
≤5,000	53
5,001 – 15,000	67
15,001 – 30,000	73
30,001 – 50,000	65
50,001 – 100,000	52
>100,000	36
Total	346

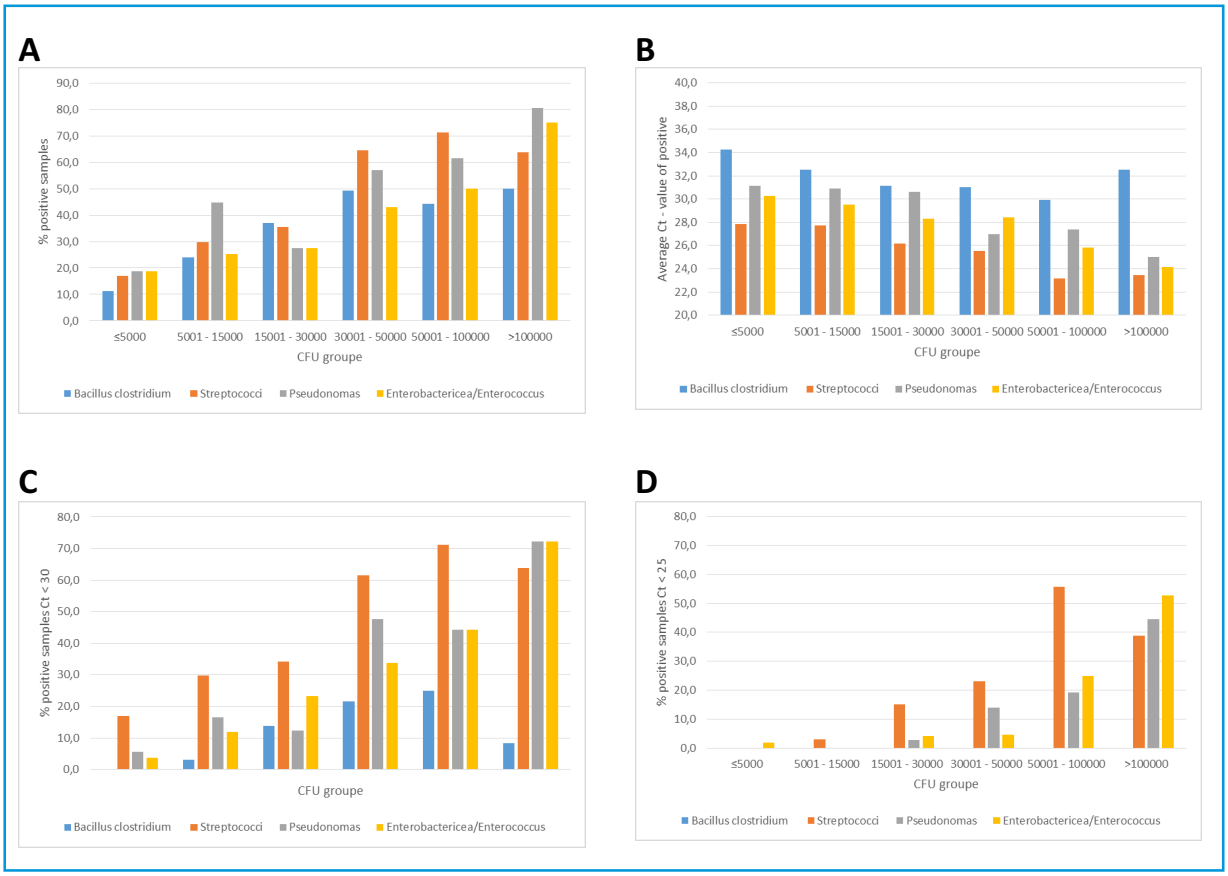

Figure a,b,c, and d: a) percent of positive samples, b) average Ct-value for positive samples, c) percent Ct-values under 30 and d) Percent Ct-values under 25 for the different groups of CFU for each of the four different targets in the qPCR test the TBC 4.

The *Pseudomonas, Streptococci and the Enterobacteriacea/Enterococcus* target showed increasing percent positive samples with higher CFU and also reduced Ct-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 and the average Ct-value were above 30 in all groups of CFU. (Figure a and b). The percent positive samples with Ct-value below 30 and 25 is shown in figure c and d. As it can be seen, we did not find many *Bacillus/Clostridia* positive samples with really low Ct-values. For the *Streptococci* they have the highest percent of samples with low Ct-values in the samples up to 100,000 CFU/mL, 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.

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 the *Pseudomonas* the *Streptococci* and the *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 Figure 1 b where the low Ct-values for these targets in samples with CFU/mL >30,000 indicates higher number of bacteria. We found *Enterobacteriacea/ Enterococcus, Pseudomonas*, and *Streptococci* in high number of bacteria (Ct <25 Figure d) in 25%, 19% and 56% of samples with CFU/mL between 50,001 - 10,0000 and 53%, 44%, and 39% in samples with CFU/mL > 100,000. Holm *et al*. (2004) found, in Danish BTM samples with > 30,000 CFU/mL, 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 mastitis primer only detects *Streptococci*, but the *Streptococci* primer can also detect *Streptococci* not so often related to mastitis e.g. *Strep. bovis*.

Our findings, that *Streptococci* is an important factor in high TBC, is in accordance Gillespie *et al*. (2012) that found strong correlation between SPC and *Streptococcus* spp. counts. Katholm *et al*., 2012 found the best correlation between TBC in bulk tank milk and Ct-values from real time PCR assays specific for *Enterococcus, Strep. uberis* and *Strep. agalactiae*, less correlation to Ct-values for *Strep. dysgalactiae*, *E.coli* and *Klebsiella*, and no correlation to *Staph. aureus*. Our findings, that the *Enterobacteriacea/ Enterococcus* is an important finding in milk samples with high CFU is in accordance with Pyz-Lukasik *et al*. (2015), they 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.

Conclusion

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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