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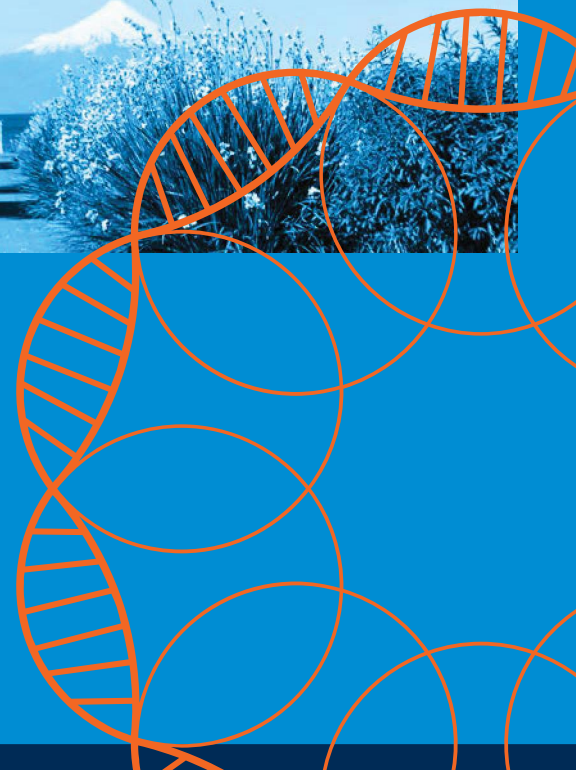
A GLOBAL PERSPECTIVE ON PERFORMANCE RECORDING AND ANIMAL IDENTIFICATION



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Interpretation of results from milk samples tested for mastitis bacteria with Mastit 4 PCR from DNA Diagnostic

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Abstract

The use of Real-time PCR tests to identify mastitis pathogens are growing, as they are faster and more sensitive than conventional bacteriological culturing, especially for *Mycoplasma* detection. Since 2014 a quantitative real-time PCR test kit (Mastit 4, DNA Diagnostic) has been commercially available. The objective of this study was to investigate the correlation between Ct values of the Mastit 4 PCR test kit for and bacterial colony forming units (CFU) in fresh milk samples for the 11 bacteria that can be detected by the Mastit 4 BDF, PCR test kit.

For each bacteria six different isolates were tested. Tenfold dilutions were made of an overnight culture of the bacteria using fresh quarter milk initially tested negative for all the studied bacteria. Culture was performed according to NMC guidelines. For each isolate CFU/ml in the initial samples were calculated from the duplicate plates of diluted samples containing between 10 and 300 colonies, and thereby the CFU/ in all the different 10-fold dilutions was calculated. All dilutions of the bacteria spiked milk samples, were tested by qPCR in the DNA Diagnostic laboratory with the Mastit 4 test using the M4BDF kit. The expected bacterial count and pathogen was blinded to the laboratory.

The dilution curves for *S. aureus*, *CNS*, *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Lactococcus lactis ssp lactis*, *E. coli*, *Klebsiella*, *Prototheca*, *Mycoplasma bovis*, and *Mycoplasma species* were produced.

Dilutions with a concentration of less than 1 CFU /0.5 ml have been excluded, since the expected number for bacteria would be less than one in the examined sample volume.

The correlation line shows an exponential relation between the Ct-value and the CFU. This was expected since the Ct value is an expression of the multiplication of bacterial DNA in the PCR-process.

The correlation lines gives a very good impression of the CFU/1/2 ml that can be expected for a measurement of a Ct-value for the different bacteria. It also gives a good impression of the Ct-value that can be expected at the detection limit of 100 CFU/ml for culture. This will correspond to a Ct value of 27-28 for *Streptococci* and Ct-value 34 for *S. aureus*.

For *S. aureus*, *CNS*, *Mycoplasma bovis* and *Mycoplasma species* a concentration of 1 CFU/0.5 ml results in a Ct-value close to 40 whereas the corresponding Ct-value for the three streptococcal species at 1 CFU/ 0.5 ml lies between 33-36. As a consequence results for streptococci with a Ct value 33 to 40 indicate a concentration of less than

1 CFU/0.5 ml. Therefore true positive samples with a bacterial concentration of less than 1 CFU/0.5 ml cannot be expected to test positive in repeated tests of the same sample. The proportion of samples with this low bacterial concentration will affect the apparent sensitivity of the test.

Keywords: Katholm, Mastit 4, PCR, CFU, Mycoplasma bovis, bacteria

Introduction

The use of Real-time PCR tests to identify mastitis pathogens are growing, as they are faster and more sensitive than conventional bacteriological culturing, especially for *Mycoplasma* detection. Since 2014 a quantitative real-time PCR test kit (Mastit 4, DNA Diagnostic) has been commercially available. In Denmark the qPCR test is mainly used as a tool for selective dry cow therapy to test DHI samples from cows prior to dry-off for subclinical intramammary infections or biannually test of bulk tank milk samples from all Danish dairy herds for *Streptococcus agalactiae* as a part of the national surveillance program.

Rattenborg et al. (2014) compared the Mastit 4 A with the Pathoproof complete 16 kit and found the agreement between the two tests to be high to moderate for *Strep. agalactiae* and moderate for *S. aureus* and *Strep. uberis*. Bennedsgaard et al. (2015) also presented correlation curves for five of the bacteria tested in this study. They also tested the correlation for culture and PCR in natural infections and found that they in all results except one showed Ct-values below the curve of the dilution experiment. This might indicate the presence of DNA from dead bacteria or that the bacteria in natural infections are more difficult to culture (intracellular *S. aureus*).

The objective of this study was to investigate the correlation between Ct values of the Mastit 4 PCR test kit for and bacterial colony forming units (CFU) in fresh milk samples for the 11 bacteria that can be detected by the Mastit 4 BDF, PCR test kit.

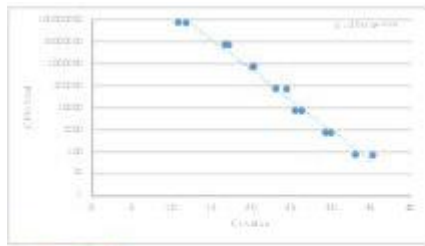
Material and methods

Of all bacteria isolates tenfold dilutions were made of an overnight culture using fresh quarter milk initially tested negative for all the studied bacteria. From *S. aureus* we tested 2 isolates, CNS 3 isolates, *Strep. agalactiae* 3 isolates, *Strep. dysgalactiae* 3 isolates, *Strep. uberis* 3 isolates, isolates, *Lactococcus lactis ssp lactis* 1 isolate, *E. coli* 3 isolates, *Klebsiella* 1 isolate Prototheca 3 isolates, *Mycoplasma bovis* 3 isolates, and *Mycoplasma* species 3 isolates

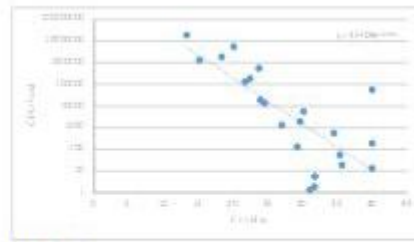
Culture was performed according to NMC guidelines. For each isolate CFU/ml in the initial samples were calculated from the duplicate plates of diluted samples containing between 10 and 300 colonies, and thereby the CFU in all the different 10-fold dilutions was calculated. All dilutions of bacteria spiked milk samples, were tested by qPCR in the DNA Diagnostic laboratory with the Mastit 4 test using the M4BDF kit standard protocol. The expected bacterial count and pathogen was blinded to the laboratory.

The dilution curves for *S. aureus*, CNS, *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Lactococcus lactis ssp lactis*, *E. coli*, *Klebsiella*, Prototheca, *Mycoplasma bovis*, and *Mycoplasma* species.were made by plotting in Excel.

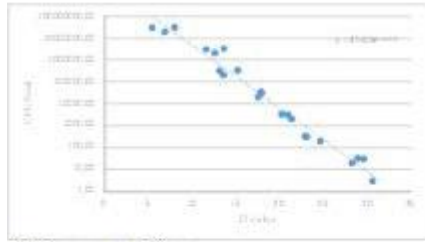
Dilutions with a concentration of less than 1 CFU /0.5 ml have been excluded, since the expected number for bacteria would be less than one in the examined sample volume.



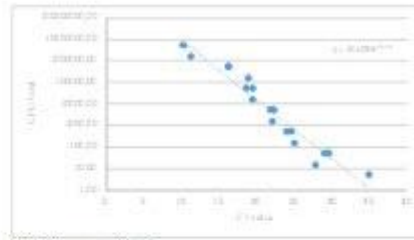
(a) *S. aureus*



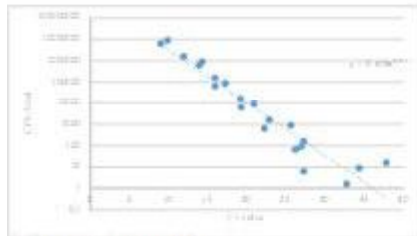
(b) CNS



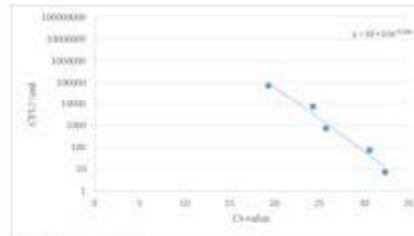
(c) *Strep. agalactiae*



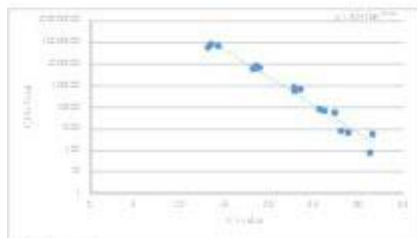
(d) *Strep. uberis*



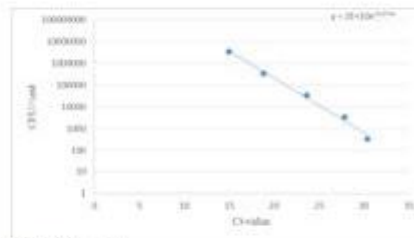
(e) *Strep. dysgalactiae*



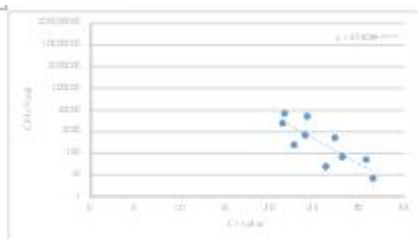
(f) *Lactococcus*



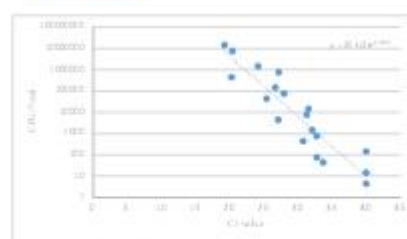
(g) *E. coli*



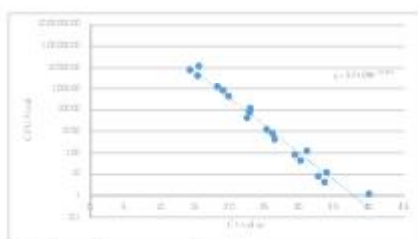
(h) *Klebsiella*



(i) *Prototheca*



(j) *Mycoplasma bovis*



(k) *Mycoplasma species*

Figure 1a-k. Correlation of CFU/ml (log scale) and Ct values for dilutions of samples spiked with *S. aureus*, CNS, *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Lactococcus lactis* ssp *lactis*, *E. coli*, *Klebsiella*, *Prototheca*, *Mycoplasma bovis*, and *Mycoplasma* species.

Results

The correlation line shows an exponential relation between the Ct-value and the CFU. This was expected since the Ct value is an expression of the multiplication of bacterial DNA in the PCR-process. Correlation figures and lines is shown in figure 1 a - k for *S. aureus*, *CNS*, *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Lactococcus lactis ssp lactis*, *E. coli*, *Klebsiella*, *Prototheca*, *Mycoplasma bovis*, and *Mycoplasma* species.

Discussion

The correlation lines gives a very good impression of the CFU/½ ml that can be expected for a measurement of a Ct-value for the different bacteria. It also gives a good impression of the Ct-value that can be expected at the detection limit of 100 CFU/ml for culture. This will correspond to a Ct value of 27-28 for streptococci and Ct-value 34 for *S. aureus*.

For *S. aureus*, *CNS*, *Mycoplasma bovis* and *Mycoplasma* species a concentration of 1 CFU/0.5 ml results in a Ct-value close to 40 whereas the corresponding Ct-value for the three streptococcal species at 1 CFU/0.5 ml lies between 33-36. As a consequence results for streptococci with a Ct value 33 to 40 indicate a concentration of less than 1 CFU/0.5 ml. Therefore true positive samples with a bacterial concentration of less than 15 CFU/0.5 ml cannot be expected to test positive in repeated tests of the same sample. The proportion of samples with this low bacterial concentration will affect the apparent sensitivity of the test.

Accreditation

Thanks to Prof, Dr. med. vet. habil. Volker Krömker and PhD. Student Nicole Wente from Hochschule Hannover, University of Applied Sciences and Arts, Hannover, for the culturing and CFU measurements of all bacteria isolates.

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