UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Master's Thesis in Veterinary Medicine

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Validation of real-time polymerase chain reaction and bacteriological culture for identification of *Streptococcus agalactiae* and *Staphylococcus aureus* in milk and on teat skin in herds with automatic milking system

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Submitted on: July 15th, 2017

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Abstract

Streptococcus agalactiae (*S. agalactiae*) and *Staphylococcus aureus* (*S. aureus*) cause intramammary infections (IMI) in dairy cows. Despite a national surveillance program for *S. agalactiae*, an increasing herd-level prevalence is seen whereas the majority of Danish dairy herds are already infected with *S. aureus.* IMI result in financial losses for the farmer and impaired animal welfare for the cow. This is why cases of mastitis should be treated as soon as possible. Valid diagnostic tests are essential for a correct diagnosis and treatment which is why more studies have estimated the sensitivity and specificity of polymerase chain reaction (PCR) against conventional bacteriological culture (BC) for the diagnosis of IMI with *S. agalactiae* and *S. aureus*. Even though *S. agalactiae* and *S. aureus* originally have been regarded as contagious mastitis pathogens, in recent years, both pathogens have been isolated from, among other things, the environment, indicating reservoirs beyond the intramammary. Therefore, validation of PCR on teat skin in the survey of potential reservoirs, and the importance of these, is of current interest. The objective of this field study was to estimate the sensitivity and specificity for the real-time PCR assay 'Mastit4' against BC for the identification of *S. agalactiae* and *S. aureus* in milk and on teat skin. Two hundred and eighty-seven cows were randomly selected from among cows with high somatic cell count (SCC) from eight Danish dairy herds with a positive status and a positive annual bulk tank milk (BTM) sample for *S. agalactiae*. Teat skin samples and aseptic milk samples from all four quarters were collected for BC whereas only right hind (RH) quarters were selected for PCR analysis. Selected positive quarters were resampled with both PCR and BC (part B) one to three weeks after the first sampling (part A). In both part A and B, sensitivity and specificity of PCR and BC for *S. agalactiae* and *S. aureus* in milk were estimated using latent class analysis (LCA) whereas sensitivity and specificity of PCR for *S. agalactiae* and *S. aureus* on teat skin were estimated relative to BC. Additionally, the agreement (kappa) between PCR and BC was estimated. For *S. agalactiae* in milk, the sensitivity and specificity of PCR were 96.4 and 93.4 %, respectively, whereas the sensitivity and specificity of BC were 82.4 and 99.7 %, respectively (kappa = 0.61). For *S. aureus* in milk, the sensitivity and specificity of PCR were 87.6 and 98.2 %, respectively, whereas the sensitivity and specificity of BC were 74.0 and 99.4 %, respectively (kappa = 0.72). Compared with BC, sensitivity and specificity of PCR on teat skin were 100 and 82.2 %, respectively, for the identification of *S. agalactiae* (kappa = 0.031) and 30.4 and 86.7 %, respectively, for the identification of *S. aureus* (kappa = 0.13). The poor agreement between PCR and BC on teat skin could indicate that the two methods do not measure the same: PCR detects viable as well as dead and inactivated bacteria whereas BC detects only viable bacteria. Further

studies are needed to investigate the importance of the teat skin as a reservoir and to improve methods for PCR and BC before introducing teat skin samples as part of herd health management. On the other hand, the agreement between PCR and BC in milk was good, and the sensitivity for diagnosing IMI caused by either *S. agalactiae* or *S. aureus* was higher for PCR than BC, indicating that PCR is more suitable for milk samples compared to BC. In the light of these results, the use of PCR for diagnosing IMI with *S. agalactiae* or *S. aureus* is therefore recommended.

Keywords: *Streptococcus agalactiae*, *Staphylococcus aureus*, dairy cows, AMS, teat skin, reservoirs, intramammary infection, mastitis, real-time PCR, bacteriological culture, sensitivity, specificity, latent class analysis, kappa

Resume

Streptococcus agalactiae (*S. agalactiae*) og *Staphylococcus aureus* (*S. aureus*) er bakterier, der begge forårsager mastitis hos køer. På trods af et nationalt overvågningsprogram for *S. agalactiae* ses en stigende prævalens på besætningsniveau, hvorimod størstedelen af danske malkekvægsbesætninger allerede er inficeret med *S. aureus*. Mastitis medfører store økonomiske tab for landmanden og nedsat dyrevelfærd for koen, hvorfor tilfælde af mastitis ønskes behandlet hurtigst muligt. Valide diagnostiske tests er essentielle for en korrekt diagnose og behandling, hvorfor flere studier har sammenlignet testegenskaberne (sensitivitet og specificitet) for polymerase chain reaction (PCR) med konventionel bakteriologisk undersøgelse (BU) til identifikation af *S. agalactiae* og *S. aureus* i mælk. Selvom både *S. agalactiae* og *S. aureus* oprindeligt er blevet betragtet som smitsomme mastitispatogener, er begge isoleret fra blandt andet miljøet de senere år, hvilket indikerer reservoirs ud over det intramammære. Validering af PCR på pattehuden er derfor aktuelt i kortlægningen af potentielle reservoirs og betydningen af disse. Formålet med dette feltstudie var at sammenligne sensitiviteten og specificiteten af den kvantitative PCR-test Mastit4 med BU for identifikation af *S. agalactiae* og *S. aureus* i både mælk og på pattehuden. Fra otte danske malkekvægsbesætninger med en positiv status og årlig tankmælksprøve for *S. agalactiae* blev 287 køer med højt celletal tilfældigt udvalgt. Fra alle fire kirtler blev der taget pattehudsprøver og aseptiske mælkeprøver til BU, mens kun højre bagkirtel blev udvalgt til PCR. Udvalgte positive kirtler blev gentestet med både PCR og BU (del B) en til tre uger efter første prøveindsamling (del A). For både del A og B blev sensitiviteten og specificiteten af PCR og BU for *S. agalactiae* og *S. aureus* på mælk udregnet ved hjælp af latentklasseanalyse, mens sensitiviteten og specificiteten af PCR for *S. agalactiae* og *S. aureus* på pattehud blev udregnet med BU som referencestandard. Herudover blev overensstemmelsen (kappa) mellem PCR og BU estimeret. For *S. agalactiae* på mælk var sensitiviteten og specificiteten af PCR henholdsvis 96.4 % og 93.4 %, mens sensitiviteten og specificiteten af BU var henholdsvis 82.4 % og 99.7 % (kappa = 0.61). For *S. aureus* på mælk var sensitiviteten og specificiteten af PCR henholdsvis 87.6 % og 98.2 %, mens sensitiviteten og specificiteten af BU var henholdsvis 74.0 % og 99.4 % (kappa = 0.72). Sensitiviteten og specificiteten af PCR på pattehuden var (sammenlignet med BU) henholdsvis 100 % og 82.2 % for identifikation af *S. agalactiae* (kappa = 0.031) og henholdsvis 30.4 % og 86.7 % for identifikation af *S. aureus* (kappa = 0.13). Den dårlige overensstemmelse mellem PCR og BU på pattehud kan indikere, at de to metoder ikke detekterer det samme: PCR detekterer levende såvel som døde og inaktiverede bakterier, mens BU kun detekterer levende bakterier. Flere studier er nødvendige for at kortlægge

pattehudens betydning og forbedre både BU og PCR, før svabring af pattehuden kan indgå som et element i besætningsrådgivning omkring yversundhed. Overensstemmelsen mellem PCR og BU på mælk var derimod god, og PCR havde en højere sensitivitet for diagnosticering af mastitis forårsaget af enten *S. agalactiae* eller *S. aureus* end BU, hvilket indikerer, at PCR er bedre egnet til analyse af mælkeprøver end BU. Brugen af PCR frem for BU som et led i diagnostikken af mastitis kan derfor anbefales.

Nøgleord: *Streptococcus agalactiae*, *Staphylococcus aureus*, malkekøer, AMS, pattehud, reservoirer, intramammære infektioner, mastitis, kvantitativ PCR, bakteriologisk dyrkning, sensitivitet, specificitet, latentklasseanalyse, kappa

Preface

This master's thesis was written as part of the Master's degree program in Veterinary Medicine at the University of Copenhagen. The master's thesis was written from February 1st to July 15th, 2017 and conducted as part of the STOPMAST project whose overall aim is to investigate the continuously high occurrence of mastitis pathogens in Denmark. The objective of this study was to validate a real-time polymerase chain reaction assay against conventional bacteriological culture for the identification of *Staphylococcus aureus* and *Streptococcus agalactiae* in milk and teat skin samples in herds with automatic milking system. Milk and teat skin samples were collected from dairy herds in Jutland, Denmark. The research is addressed to veterinarians, veterinary students, and farmers with an interest in mastitis diagnostics and reservoirs for *Staphylococcus aureus* and *Streptococcus agalactiae*.

The master's thesis was funded by the Danish Milk Levy Fund (Mælkeafgiftsfonden) and DNA Diagnostic A/S. We wish to thank Jørgen Katholm and Dennis Holt from DNA Diagnostic A/S for their help and interest in this study.

We wish to thank Ilka Christine Klaas, Line Svennesen, Yasser Mahmmod, Karl Pedersen, Michael Farre and the rest of the STOPMAST team for their help and inspiration.

Finally, we wish to thank the Danish farmers for their interest and willingness to participate and the milk quality technicians from SEGES for their help with sampling.

Submitted on July 15th, 2017

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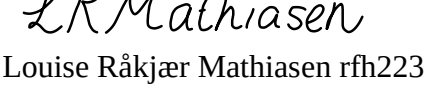

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Abbreviations

AMS	Automatic milking system(s)
BC	Bacteriological culture
BMSCC	Bulk milk somatic cell count
BTM	Bulk tank milk
CAMP	Christie Atkins Munch-Peterson
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit(s)
CI	Confidence interval(s)
CNS	Coagulase-negative staphylococci
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
IMI	Intramammary infection(s)
IMM	Intramammary application
LCA	Latent class analysis
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MC	Monte Carlo
MCMC	Markov Chain Monte Carlo
mRNA	Messenger ribonucleic acid
NMC	National Mastitis Council

NPV	Negative predictive value
PCI	Posterior credibility interval(s)
PCR	Polymerase chain reaction
PPV	Positive predictive value
qPCR	Quantitative polymerase chain reaction
RH	Right hind
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
S. dysgalactiae	*Streptococcus dysgalactiae*
S. uberis	*Streptococcus uberis*
SCC	Somatic cell count
Se_{BC}	Sensitivity of bacteriological culture
Se_{PCR}	Sensitivity of polymerase chain reaction
Sp_{BC}	Specificity of bacteriological culture
Sp_{PCR}	Specificity of polymerase chain reaction
ST	Sequence type(s)

Introduction

Streptococcus agalactiae (*S. agalactiae*) and *Staphylococcus aureus* (*S. aureus*) are bacteria causing intramammary infections (IMI) in dairy cows. In 2010, polymerase chain reaction (PCR) on bulk tank milk (BTM) samples detected *S. aureus* and *S. agalactiae* in 91 and 7 % of Danish herds, respectively (Katholm et al. 2012). Based on bacteriological culture (BC), the within-herd prevalence of *S. aureus* ranged from 13 to 33 % (Mahmmod et al. 2013c) whereas the within-herd prevalence of *S. agalactiae* ranged from 1.6 to 21.7 % (Mahmmod et al. 2015). Even though *S. agalactiae* is the only mastitis pathogen regulated by law, and a surveillance program has existed since 1954, the herd-level incidence and prevalence in Danish dairy herds have been increasing from 2000 to 2009 as indicated by Mweu et al. (2012).

IMI with *S. aureus* and *S. agalactiae* result in impaired udder health and milk quality because of a high somatic cell count (SCC) (Katholm et al. 2012). Treatment of *S. aureus* has low cure rates (Keefe 2012), and costs related to a single case of *S. aureus* mastitis are high, estimated to €570 by Sørensen et al. (2010). Today, IMI make up a high share of the total usage of antibiotics in Danish herds (Bager et al. 2016), and therefore, effective control of these mastitis pathogens is important. Even though both bacteria are categorized as contagious mastitis pathogens, environmental reservoirs are described in the scientific literature (Zadoks et al. 2011; Jørgensen et al. 2016). The importance of these environmental reservoirs is still discussed and needs further investigation, especially for *S. agalactiae*.

The development of the dairy industry in Denmark goes towards larger herds which has led to the evolvement of new milking systems like automatic milking system (AMS). With AMS, the daily handling of the udder by the milker is eliminated. Because the transmission of contagious mastitis pathogens occurs primarily during milking, a difference in the prevalence of *S. aureus* and *S. agalactiae*, isolated from both milk and teat skin in AMS compared to conventional milking systems, is likely. In addition, many Danish herds with AMS are *S. agalactiae* positive and have problems with eradication (Katholm 2010).

So far, BC has been the reference standard for identification of mastitis pathogens. But due to the higher sensitivity (Koskinen et al. 2010) and speed (Smith 2009), PCR is gaining more footage.

Studies comparing the sensitivity and specificity of real-time PCR and BC for the diagnosis of IMI with either *S. aureus* or *S. agalactiae* in milk samples have shown a higher sensitivity of PCR compared to BC (Mahmmod et al. 2013a; Mahmmod et al. 2013b). To our knowledge, no

published studies have estimated the sensitivity and specificity of real-time PCR and BC for the identification of *S. aureus* and *S. agalactiae* in milk and teat skin samples from Danish dairy herds with AMS.

This master's thesis was carried out as part of the STOPMAST project whose overall aim is to investigate the continuously high occurrence of mastitis pathogens in Denmark. The bovine teat skin may be an important reservoir, therefore validation of diagnostic tests, like PCR, is important. PCR tests on teat skin samples could turn out to be a feasible tool in udder health management and an important part in controlling *S. agalactiae* and *S. aureus* mastitis.

Aims and hypotheses

The objective of this field study was to estimate the sensitivity and specificity of the commercial quantitative PCR (qPCR) assay Mastit4 (DNA Diagnostic A/S, Risskov, Denmark) and BC for the identification of *S. aureus* and *S. agalactiae* in milk and on teat skin. The field study was divided into two parts, A and B, so that cows positive in part A were resampled in part B, to assure a satisfactory proportion of positive samples. Furthermore, part B involved two different sampling procedures.

Hypotheses:

- *S. agalactiae* and *S. aureus* can be isolated from teat skin and milk and can be detected by both PCR and BC
- The Mastit4 qPCR Assay has a higher sensitivity compared to BC for both *S. agalactiae* and *S. aureus* in milk and teat skin samples
- There is a high correlation between cycle threshold (Ct) values for PCR and colony forming units (CFU) for BC for both *S. agalactiae* and *S. aureus* in milk and teat skin samples

Bovine *S. agalactiae* and *S. aureus* mastitis

Prevalence

The herd-level prevalence of *S. agalactiae* and *S. aureus* differs. Katholm et al. (2012) detected *S. aureus* in 91 % of the Danish herds whereas *S. agalactiae* was detected in only 7 %. Similar herd-level prevalence of *S. aureus* has been reported from countries other than Denmark.

In Germany, Tenhagen et al. (2006) found *S. aureus* in 90 % of the 80 investigated herds, and in Belgium, *S. aureus* was found in 86 % of the herds with a mean within-herd prevalence of 3.2 %, ranging from 0 to 40.3 % (Piepers et al. 2007). The mean within-herd prevalence of *S. aureus* in six Danish herds was 25 %, ranging from 13 to 33 % (Mahmmod et al. 2013c). The mean quarter-level prevalence of *S. aureus* was ranging from 3.1 % in Belgian herds (Piepers et al. 2007) to 5.7 % in German herds (Tenhagen et al. 2006). In subclinical samples, the prevalence of *S. aureus* was ranging from 5.2 (Bradley et al. 2007) to 28.2 % (Gianneechini et al. 2002) whereas in clinical samples, the prevalence was ranging from 3.3 (Bradley et al. 2007) to 37.5 % (Gianneechini et al. 2002).

The herd-level prevalence of *S. agalactiae* shows more variation than of *S. aureus*. In Germany, Tenhagen et al. (2006) found *S. agalactiae* in 29 % of the 80 investigated herds whereas Piepers et al. (2007) found *S. agalactiae* in only 5.3 % of Belgian herds with a mean within-herd prevalence of 0.1 %, ranging from 0 to 4.2 %. *S. agalactiae* was not isolated from neither 480 clinical samples from 97 British herds (Bradley et al. 2007) nor 2174 subclinical samples from 49 Dutch herds (Sampimon et al. 2009). The mean within-herd prevalence of *S. agalactiae* in six Danish herds was 7.8 %, ranging from 1.6 to 21.7 % (Mahmmod et al. 2015). Furthermore, the mean quarter-level prevalence of *S. agalactiae* was 2.8 %, ranging from 0.4 to 7.8 % (Mahmmod et al. 2015). Outside Denmark, the mean quarter-level prevalence of *S. agalactiae* ranged from 0.1 % in Belgium (Piepers et al. 2007) to 0.7 % in Germany (Tenhagen et al. 2006). In subclinical and clinical samples, the prevalence of *S. agalactiae* was 5.1 and 5 %, respectively (Gianneechini et al. 2002).

Impact

Clinical mastitis, characterized by marked oedema and tenderness in the udder, can be an acute and painful condition that affects animal welfare (Eshraghi et al. 1999). Furthermore, cases of mastitis have a great economic impact, implying unrealised production potential, discarded milk,

decreased milk quality, culling of severely affected cows, occurrence of other diseases due to complications, and expenses for drugs, veterinarian working hours, and labor for the farmer (Halasa et al. 2007; Huijps 2009). The expenses can vary greatly from farm to farm, and case to case, depending on the severity of the mastitis case and the management status on the farm (Huijps 2009).

The SimHerd simulation model predicts a case of mastitis to cause a yield loss of 7.7 % of lactation yield, depending on which time in lactation the disease occurs. The prediction is an overall estimate regarding both clinical and subclinical mastitis (Østergaard et al. 2005; Ettema 2015). The model expects mastitis to have direct effects on feed intake, body weight, milk yield, SCC, subsequent mastitis cases within cows and in herd mates, voluntary and involuntary culling, mortality, and milk withdrawal (Østergaard et al. 2005). Sørensen et al. (2010), using the same SimHerd model but with specific pathogens, estimated one case of *S. aureus* mastitis, adjusted for the expected type of mastitis (most frequently subclinical), to cost €570.

To prevent or cure mastitis, antibiotics are routinely used in herd management, both as dry cow therapy and for treatment of mastitis cases. In Denmark, the majority of antimicrobials administered parenterally for dairy cows is prescribed for mastitis, mostly being beta-lactamase sensitive penicillins (Bager et al. 2016). Large-scale use of antibiotics may lead to increased prevalence of antibiotic resistance which further can lead to subsequent transfer of bacteria or resistance genes to humans or human pathogens (Teuber 1999).

Antibiotic resistance differs among countries. In Turkey, Kenar et al. (2017) found 82 out of 83 *S. aureus* isolates from bovine subclinical mastitis to be resistant to at least one of the 16 antibiotics studied. Fifty-three isolates were found to be resistant to penicillin. Among European countries, year 2002 – 2004, isolates of *S. aureus* from all involved countries, including Denmark, showed low frequency of resistance. A higher level of penicillin resistance (> 10 %) compared to other types of antibiotics was noted in almost all countries, except Norway (Garmo et al. 2010), France, and Sweden (Hendriksen et al. 2008). Because of the risk of transfer of resistance genes and evolvement of antibiotic resistance, proper control of bovine mastitis is important.

Intramammary infection

Both *S. aureus* and *S. agalactiae* cause a low-grade persistent type of infection which is typically subclinical at cow-level. They cause changes in milk composition and an increase in SCC (Harmon 1994).

Whether cows with *S. aureus* or *S. agalactiae* mastitis shift from subclinical to clinical infection depends on many factors, e.g. bacterial load, type of bacteria strain, and the status of the immune system. In general, mastitis is caused by bacterial invasion of the udder through the teat canal. The teat canal acts as the first barrier and is sealed between milkings by a keratin plug. Together with the sphincter muscles and the teat end, this keratin plug works as a physical barrier, preventing penetration of bacteria. At milking, the teat end sphincter opens and requires two hours post milking to contract (Rainard & Riollet 2006).

If bacteria manage to go through the teat canal, the few somatic cells present in the udder will try to combat this IMI immediately. Bacteria and leucocytes of the infected quarter will release chemoattractants for leukocytes, and neutrophils from the bloodstream will move rapidly to the udder and cause an immediate increase in somatic cells in the milk (Suriyasathaporn et al. 2000; Rainard & Riollet 2006). If bacteria are combated, only a mild inflammatory episode will be required to restore a healthy gland. Sometimes, the innate immune system, in the shape of neutrophils, is not enough, and bacteria will multiply. This results in a prolonged immune response where different arriving cell types will release cytokines which, among other factors, will cause clinical signs of mastitis (Suriyasathaporn et al. 2000). According to Harmon (1994), clinical signs of mastitis are characterized by swelling or pain in the udder, abnormal appearance of milk, and, in some cases, increased temperature, lethargy, anorexia, and even death.

Cows with *S. agalactiae* mastitis usually have subclinical mastitis, characterized by normal milk but elevated SCC, and they shed high levels of bacteria into the bulk tank. Occasionally, the cow may progress from subclinical to clinical mastitis which will include clinical signs like udder swelling and abnormal milk (Maroney 2005). These clinical cases can be observed in herds with good control of contagious mastitis, evidenced by low (< 150.000 cells/ml) bulk milk SCC (BMSCC) since the low appearance of leukocytes in the udder will extend the combat of mastitis pathogens (Suriyasathaporn et al. 2000; Zadoks & Fitzpatrick 2009).

IMI with *S. aureus* occur more frequently in herds compared to IMI with *S. agalactiae* as described above. In an experimental challenge, Shoshani & Berman (1998) found *S. aureus* mastitis to be subclinical, characterized by elevated SCC, no clinical signs, and no changes in milk or milk yield. Barkema et al. (1998) cultured *S. aureus* most often from herds with high BMSCC. In herds with low BMSCC, cases of clinical mastitis with *S. aureus* were found to be more severe compared to clinical mastitis in herds with high BMSCC (Barkema et al. 1998).

Treatment

S. agalactiae has a high rate of successful treatments for both subclinical and clinical cases. Reyes et al. (2015) found 82.4 % of subclinically infected cows to be bacteriologically cured using intramammary application (IMM) of ampicillin-cloxacillin and 65.8 % of subclinically infected cows to be bacteriologically cured by systemic treatment with penethamate hydriodide. These findings agree with the review article from Keefe (1997), reporting lactational therapy cure rates for IMM treatment between 84 and 100 %. Higher cow SCC at time of treatment had a trend for lower cure rates for both IMM and systemic treatment (Reyes et al. 2015). Sérieys et al. (2005) found seven clinical cow cases with *S. agalactiae*, of which four cows were bacteriologically cured. Due to few cases in this study, more knowledge about cure rates for clinical cases is needed.

S. aureus has a low rate of successful treatments during lactation. *S. aureus* can withstand killing by neutrophils (Mullarky et al. 2001), invade mammary epithelial cells (Hensen et al. 2000), form microabscesses (Keefe 2012), and promote biofilm formation (Schönborn et al. 2017) which perhaps can explain the low rate of successful treatments.

Pyörälä & Pyörälä (1998) found that 34 % of clinical cases of *S. aureus* mastitis were bacteriologically cured using parenteral treatment with procaine penicillin G, with cure rates higher in cows in their first lactation. Subclinical mastitis with *S. aureus* has a low cure rate with treatment during lactation as well. Sol et al. (1997) found that 34 % of subclinical cases of *S. aureus* mastitis were bacteriologically cured. Deluyker et al. (2005) found that subclinical cases of *S. aureus* had a bacteriologically cure rate of 56 % after two days with IMM treatment and 86 % after eight days with IMM treatment. Cure rates of *S. aureus* mastitis decrease with increasing age of the cow, increasing SCC, increasing duration of infection, increasing bacterial colony counts in milk before treatment, and increasing number of quarters infected (Barkema et al. 2006).

Shedding of *S. aureus* and *S. agalactiae* happens in a cyclic pattern from infected quarters which can complicate diagnostics and thus treatment (Sears et al. 1990; Shoshani & Berman 1998; Katholm 2010; Keefe 2012). Sol et al. (1997) found that non-cured cases of subclinical mastitis continued shedding and became a permanent source of infection for other cows (Lam et al. 1996).

Reservoirs

Given that *S. agalactiae* and *S. aureus* are generally regarded as highly contagious bacteria, the intramammary reservoir is the primary reservoir for both pathogens. Alternative reservoirs have been described for both pathogens. Jørgensen et al. (2016) detected *S. agalactiae* in rectal swabs

and swabs from, among other things, the AMS, floors, and equipment with BC, suggesting that the bovine gastrointestinal tract and farm environment are reservoirs of *S. agalactiae*. To the best of our knowledge, the bovine teat skin as a reservoir of *S. agalactiae* has not yet been considered. On the other hand, the bovine teat skin as a reservoir of *S. aureus* is widely described, and its role in the pathogenesis of IMI is still being discussed.

Several studies show that colonization of teat skin with *S. aureus* increases the risk of IMI with *S. aureus* (Roberson et al. 1994; Haveri et al. 2008; Piccinini et al. 2009; da Costa et al. 2014). Da Costa et al. (2014) showed that quarters with teat skin colonization with *S. aureus* were 4.5 times more likely to be diagnosed with *S. aureus* IMI than quarters with no teat skin colonization. However, Zadoks et al. (2002) did not find the teat skin to play an important role in the pathogenesis of IMI with *S. aureus*. The role of teat skin in the pathogenesis of IMI is thus still uncertain and gives rise to the following question: Does the teat skin pose a potential source of IMI, or do IMI result in teat skin contamination (Zadoks et al. 2011)? Besides on teat skin, *S. aureus* has been detected in non-bovine animals, humans, flies, and in the farm environment (Zadoks & Fitzpatrick 2009; Zadoks et al. 2011).

Detection methods

Today, in Denmark, diagnosis of *S. aureus* and *S. agalactiae* mastitis is mostly based on BC of aseptically taken milk samples in the laboratory of veterinary practices, using primarily blood agar, chrome agar, and penicillin agar, confirmation mostly based on morphology (Geijer 2011). According to the annual mastitis diagnostics proficiency test from 2016, all falsely identified mastitis pathogens could have been correctly identified using only one biochemical test, indicating limited use of other confirmatory options apart from morphology in Danish veterinary practices (Astrup et al. 2016). It should be noted that only 41 persons participated in the proficiency test in 2016, and that these participants, including the 10 participants from the master's thesis of Geijer (2011), most probably represented veterinary practices with good mastitis diagnostic routines.

Since 2009, it has been possible to order cow-level PCR tests as part of routine milk testing with the purpose of directing dry cow therapy or diagnose mastitis cases (Farre 2017, personal communication). In 2014, more than 68.000 PCR tests as part of routine milk testing were done (Hansen 2015). In the following section, basic principles behind BC and PCR will be described, followed by an introduction to confirmatory options, e.g. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and agglutination test.

Bacteriological culture

Principle

The principle of BC is the isolation and identification of viable bacterial cells. Bacteria reproduce by binary fission i.e. the separation of the bacterial cell into two daughter cells. The time required for a single bacterial cell to produce two daughter cells is called the generation time and is influenced by both genetic and nutritional factors (Quinn et al. 2011). Although the generation time for some bacteria is short, BC is slow. The conditions for bacterial growth are, besides nutrients, influenced by temperature, pH, osmotic pressure, atmospheric composition, and the availability of moisture (Quinn et al. 2011). The specific conditions for growth differ between different bacteria, and BC makes use of this knowledge in the isolation and identification of bacteria. Usually, both morphological and biochemical characteristics assist in the conventional interpretation of bacterial colonies.

Morphological and biochemical characteristics of *S. agalactiae*

S. agalactiae is a facultative anaerobic, non-motile, catalase-negative Gram-positive coccus. On calf blood agar, the colonies of *S. agalactiae* appear moist and translucent, are grey to white, and most of them produce beta-hemolysis (Daignault et al. 2003; NMC 2004) seen as a clear zone around colonies whereas a few produce no hemolysis (gamma-hemolysis). Christie Atkins Munch-Peterson (CAMP) factor is produced causing complete hemolysis of calf blood erythrocytes under effect of *S. aureus* beta-toxin, this complete hemolysis is called CAMP reaction. *S. agalactiae* does not produce aesculin hydrolysis why colonies appear greyish with CAMP reaction on modified Edward's medium with beta-toxin. Other streptococci species will appear black because of their ability to hydrolyze aesculin (Quinn et al. 2011).

Morphological and biochemical characteristics of *S. aureus*

S. aureus is a facultative anaerobic, non-motile, catalase-positive and coagulase-positive Gram-positive coccus. On calf blood agar, the colonies are creamy, golden yellow to greyish-white and produce both alfa-hemolysin and beta-hemolysin causing a double hemolysis. On calf blood agar, the alfa-hemolysis is a narrow zone of complete hemolysis around the colonies whereas the beta-hemolysis is a wider zone of incomplete hemolysis (Quinn et al. 2011). Colonies of *S. aureus* will appear pink to orange on the selective medium SA*Select*™ (SA*Select*™ 2015).

Conventional confirmation

Confirmation of bacteria can be done in more ways. Conventional bacterial confirmation consists, among others, of several biochemical tests where confirmation is based on the presence or absence

of certain biochemical properties of the bacteria. Besides biochemical tests, serological tests, like agglutination tests, are used, and confirmation is based on the presence or absence of agglutination between the bacterial antigens and corresponding antibodies. MALDI-TOF MS, a recent technique for identification or confirmation, is discussed later.

Latex agglutination: Latex agglutination is a serological test. In general, the reaction between an antigen and an antibody results in visible clumping called agglutination (vlab.amrita.edu 2011). Latex agglutination is the reaction between an antigen (or antibody) and an antibody (or antigen) coated on the surface of latex particles (vlab.amrita.edu 2011). Agglutination tests can be both qualitative and quantitative. PathoDxtra^{TM} Strep Grouping Kit (Thermo Scientific) is a qualitative latex agglutination test for the identification of clinically important streptococci. The latex particles are coated in the relevant specific antibodies which agglutinate with any corresponding streptococcal antigen extracted from the bacterial cell wall (PathoDxtra Strep Grouping Kit 2012).

The advantages of the PathoDxtra^{TM} Strep Grouping Kit are the speed and convenience. There is no need for incubation, and both procedure and interpretation are simple. If the test is positive, a granular agglutination pattern is observed in 60 seconds, otherwise the test is negative with a uniform, milky appearance (PathoDxtra Strep Grouping Kit 2012).

Bacterial load

One of the advantages of BC is the detection of only viable bacterial cells. Viable cell counts are determined primarily by colony counting which can be done using different techniques (Quinn et al. 2011). The number of viable bacteria is referred to as CFU because it is impossible to differentiate single bacterial cells from adjoining bacterial cells. The total cell count i.e. both viable and nonviable bacterial cells can be determined by microscopic counting, e.g. counting chamber, and additional counting methods, e.g. electronic counting and real-time PCR (Høiby et al. 2009; Quinn et al. 2011). Another advantage of BC is the interpretation of the agar plates, making it possible to detect contamination or mixed cultures. Pure cultures are very important for the identification and further analyses of bacteria (Høiby et al. 2009), otherwise the sensitivity may be underestimated.

Polymerase chain reaction

Conventional PCR

PCR is one of several molecular diagnostic methods using the properties of the chemical structure of deoxyribonucleic acid (DNA) for analytical purposes (Quinn et al. 2011). PCR is a fast and

accurate diagnostic tool which advances a correct diagnosis and thus treatment (Smith 2009). The disadvantages are the expenses and the risk of false positives due to a high sensitivity (Høiby et al. 2009; Smith 2009). The overall principle of PCR is the amplification of a target sequence of DNA by multiple cycles of transcription. Each cycle consists typically of three steps at different temperatures (Smith 2009). In the first step, known as denaturation, the two DNA strands are separated. In the second step, known as annealing, the temperature is lowered allowing two synthetic DNA primers to bind to opposite template DNA strands. In the third step, known as extension, the temperature is increased to typically 74°C, initiating the synthesis of new DNA strand by the enzyme DNA polymerase. In conventional PCR, this cycle is repeated up to 30 times, and the amplicon is detected by conventional agarose gel electrophoresis (Quinn et al. 2011).

Real-time PCR

Real-time PCR or qPCR is a quantitative method measuring the amount of the target sequence during every single cycle in real time (Smith 2009). Real-time PCR consists of the same three steps as conventional PCR (Quinn et al. 2011) but differs by the way of detecting the amplicon. In real-time PCR, the amplicon is detected using fluorescence: A *Taq*Man DNA probe is labelled with both a reporter dye and a quencher dye. Due to the proximity of the two dyes, the quencher dye absorbs the fluorescent signal of the reporter dye. The *Taq*Man DNA probe binds to the denatured DNA strand. If the primers are extended by the *Taq* DNA polymerase, the probe will be hydrolyzed, thus separating the two dyes. The fluorescent signal of the reporter dye will no longer be absorbed by the quencher dye, showing an increase in fluorescence (Smith 2009; Quinn et al. 2011). As the number of amplicons increases during the real-time PCR, the fluorescent signal produced also increases (Høiby et al. 2009; Quinn et al. 2011). The fluorescent signal produced from the sample must be differentiated from the fluorescent signal produced by the background: When plotting the fluorescent signal against the cycle number, only samples above a certain fluorescence threshold are defined as positive (Quinn et al. 2011). The Ct value represents the number of PCR cycles required to reach this particulate threshold. The fewer PCR cycles required to reach the threshold, the more bacterial DNA will be present in the sample.

Multiplex PCR: An infection can often be caused by several different bacteria hence the disease-causing bacterium is unknown. The advantage of real-time PCR, besides its speed and sensitivity, is its ability to run the reaction with more than one bacterium of interest by the addition of more specific primers and probes (Høiby et al. 2009). Every specific probe is labelled with a specific reporter dye (Høiby et al. 2009). During the PCR, the target sequence(s) from one or more disease-

causing bacteria are amplified and differentiated based on the different reporter dyes (Høiby et al. 2009). This is called multiplex PCR.

Reverse transcription PCR

One of the disadvantages of PCR is the detection of both viable and nonviable cells (Quinn et al. 2011). This disadvantage can be overcome by reverse transcription PCR (RT-PCR) where amplification is based on ribonucleic acid (RNA) instead of DNA, thus detecting only viable cells (Quinn et al. 2011). At first, messenger RNA (mRNA) is isolated from the cells and after addition of the first primer, synthesis of a complementary DNA (cDNA) strand is done by the enzyme reverse transcriptase (Alberts et al. 2010). The two strands (mRNA and cDNA) are separated allowing the second primer to bind to the cDNA which is then amplified through many cycles of PCR (Alberts et al. 2010).

MALDI-TOF MS

MALDI-TOF MS is a relatively new mass analysis technique for the identification of bacteria and fungi. The overall principle is the differentiation of bacterial species based mainly on their protein content. Colony material from the bacterium of interest is placed on a metal sample slide followed by the addition of a matrix. A laser light is focused on the metal sample slide, and the laser energy, in the form of UV light, is absorbed by the matrix molecules. This results in ablation of both matrix and analyte molecules from the surface of the sample followed by ionization (ARCC Chem 2016). The ions formed are accelerated by a high voltage supply and separated by mass. The time it takes for the ions to reach the detector (the Time-Of-Flight) is recorded and converted into mass i.e. smaller molecules reach the detector faster than larger molecules. Based on the masses from all the detected molecules, a protein profile or spectrum is generated for each bacterium (Iowa State University n.d.). To determine the bacterial species, the generated spectrum is compared to reference spectra in a database.

The technique is fast and easy. Identification takes around 10 minutes and the metal sample slide has room for 96 samples which are analyzed at the same time. Like conventional bacterial confirmation, the technique does not allow the identification of multiple bacteria in the same sample hence monocultures are required for analysis (Kofod et al. 2011).

Prevention, eradication, and control

Mastitis Control Program

Since *S. agalactiae* and *S. aureus* have mainly been regarded as contagious mastitis pathogens, the same prevention and control measures basically exist for both pathogens. In 1966, Neave et al. presented a plan for controlling udder disease. The plan was built on two principles: 1) reducing the amount of new cases of subclinical and clinical mastitis, and 2) shortening the duration of infection (Neave et al. 1966). The Mastitis Control Program of the National Mastitis Council (NMC) is a continuation of the original plan from 1966. It consists of 10 points or areas, in which action is important, and its principles are grossly the same as for the 1966 plan (NMC 2000).

For contagious mastitis pathogens such as *S. aureus* and *S. agalactiae*, it is important to prevent the spread of bacteria at milking time by introducing good milking hygiene. For conventional milking systems, this includes cleaning teats with individual paper towels, using gloves at milking, assuring proper milk let down by fore-stripping and applying post milking teat disinfection with an effective teat disinfectant (Neave et al. 1966; NMC 2000; Keefe 2012).

Prevention and control strategies not related to milking time include antibiotic dry cow treatment or, if necessary, culling of cows. Within- and between-herd biosecurity, which involve identification and segregation of infected cows, testing before introducing new animals, and generally avoiding introduction of infected animals to the herd, are also important (NMC 2000; Barkema et al. 2006; Keefe 2012).

Mastitis control in AMS

With AMS, other challenges and opportunities exist when it comes to mastitis control and management. After introduction of AMS in Denmark in 1998, bulk milk total bacterial count and SCC increased on a national basis (Rasmussen & Larsen 2003). In general, herd sizes are larger in herds with AMS compared to herds with conventional milking systems which often results in fewer staff hours spent per cow, further leading to poorer detection rates of new mastitis cases. Cows in herds with AMS are milked more frequently and with shorter intervals, increasing the time in risk for IMI since teat end sphincter muscles require hours to close post milking, as mentioned. Pre-milking teat cleaning is done without the visual control by the milker, and cleaner barn environment is therefore required. Cows are milked quarter-based which decreases the spread of bacteria between teats and reduces the occurrence of over milking. On the other hand, cow-to-cow transmission is no longer possible to control through milking order, thus segregation of the

herd into groups of cows with the same disease status and proper cleaning of liners between milkings are important in AMS herds (Hovinen & Pyörälä 2011).

In AMS herds, electrical conductivity (EC) of the milk is a tool for detection of cows and quarters with clinical and subclinical mastitis. EC measures the concentration of electrolytes, increasing with IMI (Shoshani & Berman 1998). The sensitivity of EC as a diagnostic tool for subclinical mastitis is low due to influences such as stage of lactation, breed, milking intervals, and oestrus (Lansbergen et al. 1994; Hamann & Zecconi 1998). Taking milk yield into account may increase the sensitivity (Shoshani & Berman 1998).

Surveillance program for *S. agalactiae*

In 1954, a national (Danish) surveillance program for *S. agalactiae* was initiated and later followed by an eradication program in 1963. In 1988, the eradication program became voluntary, and in 1995, the economic support of laboratory costs ended. From 1995 until today, BTM samples have been examined every year. Today, the consequences towards infected herds are a ban on participating in cattle shows with lactating cows and the duty to inform about the status of the herd to all in contact as described in 'Bekendtgørelse om overvågning af mastitis' (*Bek. 225 af 17/03/2005*) (Andersen et al. 2003; Katholm & Rattenborg 2009). Since 2009, annual tests on BTM have been done using PCR (Farre 2017, personal communication).

To obtain negative or free status, the herd should have four negative BTM samples in a row taken with minimum 30 days interval. Another way to obtain negative status is to take quarter samples from every cow on the same day (all with negative results) as described in 'Vejledning om B-streptokok mastitis hos kvæg' (*Vejl. 9339 af 1/1/2005*). These quarter samples will most often be taken automatically at routine milk recordings and analyzed with a multiplex real-time PCR test. Some limitations with this sampling protocol exist since *S. agalactiae* carryover from cow to cow through milking equipment in both AMS and conventional milking system is a well-known fact (Løvendahl & Bjerring 2006; Mahmmod et al. 2014). This leads to overestimation of the prevalence of infected cows, and false positive results can lead to inaccurate diagnosis and treatment or even culling of potentially healthy cows. Cross-contamination can also occur with BTM samples (Andersen et al. 2003).

The success of the surveillance program can be discussed. Herd-level prevalence of *S. agalactiae* was 20 to 30 % in the 1950s. When the national eradication program was established in 1963, the prevalence dropped to 2 % in 1979 and through the 1980s and 1990s. In 2009, the prevalence increased to 6.1 %, an ongoing process since 2000 (Zadoks et al. 2011; Katholm et al. 2012). The

increase in prevalence can be explained by several factors, e.g. herd sizes have increased through the years which makes it harder to eradicate *S. agalactiae*; since 1995, it was no longer compulsory to eradicate; and since 2005, there was no longer limitations on trading of animals. All explanations causing lower motivation for eradication (Katholm & Rattenborg 2009). The introduction of PCR testing in 2009 has increased the sensitivity for analysis on BTM, and more herds might have been identified as positive. Also, the fact that *S. agalactiae* seems to have an environmental reservoir, and the fact that the human reservoir makes up a source of infection (Zadoks et al. 2011; Lyhs et al. 2016), make the eradication harder with a surveillance program built on contagious pathogen behavior.

Materials and methods

Part A

Herd selection

Eight Danish dairy herds were selected as part of a Ph.D. project by Line Svennesen investigating the association between *S. agalactiae* and *S. aureus* present on teat skin and in milk and Yasser Mahmmod's postdoc concerning coagulase-negative staphylococci (CNS) species on teat skin and in milk (both included in the STOPMAST project). The herds had to have an AMS, a minimum of 150 cow-years (the number of cow-years equals the number of cows with 365 days of feeding), and a positive status for *S. agalactiae* per November 2016 which was confirmed positive (Ct < 40) for *S. agalactiae* at the latest annual test on BTM. Twenty-three herds met these criteria and were all contacted by letter – eight herds responded positively and were all included in the study. In January 2017, all eight herds were retested on BTM for *S. agalactiae* three times with at least one week's interval with both the Pathoproof^{TM} Mastitis PCR Assay (Finnzymes Oy, Espoo, Finland) and the Mastit4 qPCR Assay. Both PCR tests were used to increase the sensitivity. All herds had to be positive (Ct value < 32) in minimum one of the two PCR tests and minimum two out of three testing. All eight herds met these criteria.

Cow selection

From each herd, between 30 and 40 cows were included in the study. The cows were selected based on a high (> 200.000 cells/ml) SCC at last milk recording. If ≤ 40 cows had a high SCC at last milk recording, all cows were included in the study. If > 40 cows had a high SCC, 40 cows were randomly selected using PROC SURVEYSELECT in SAS software. Often, all cows with high SCC were included in the study. From herd 1, 32 cows were included, whereas 40 cows were included from each of the remaining herds. Time from last milk recording to sample visit varied from five to 33 days. Farmers were asked to exclude any treated animals during this period. Dry cows were also excluded.

Quarter selection

All four quarters were sampled for BC but due to economic constraints, only RH quarters were selected for analysis with PCR. It was assumed that the probability of teat skin colonization increased if the corresponding quarter was infected intramammarily. Dry quarters were excluded.

Sample collection

Before the sampling period, a training session was carried out on February 8th, 2017 at I/S Højlandsgården, Haslev. For four hours, sampling of teat skin and milk following the protocol was practiced.

The sampling took place from February 14th to April 18th, 2017. Quarter milk samples were mostly taken by milk quality technicians from SEGES (Landbrug & Fødevarer F.m.b.A, Aarhus N, Denmark) whereas teat skin samples were all taken by Line Svennesen (Ph.D. student) or the authors of the master's thesis. Risk of contamination between milk samples and teat skin samples was thus kept at a minimum.

Teat skin samples: A new pair of gloves was worn for each cow. The teats were prepared with dry paper towels, at least one for each teat, until the teats were visually clean. The teat skin sample was taken with the wet-dry method described by Paduch & Krömker (2011), starting with the closest quarters. A sterile rayon swab (Dakla Pack ®) was immersed into the corresponding sterile tube (15 ml centrifuge tube) with two ml of ¼ Ringer's solution, and the wet swab was rolled 360° around the teat canal orifice at a distance of one cm and then broken off into the Ringer's tube so that the swab left in the tube had not been touched. A second swab (dry) was taken in the same way but without being immersed into the Ringer's solution before sampling. Both swabs were left in the tube after sampling. To facilitate milk letdown, the teat was fore-stripped when all four quarters were sampled.

Milk samples: Milk samples were taken according to the standard procedures described by the NMC (NMC 1999). Gloves were cleaned with alcohol or a new pair of gloves was worn for each cow. The teat end was disinfected with cotton moistened in 70 % alcohol, starting with the quarters most far away, at least one cotton pad per quarter, until the cotton pad was visually clean. The first two to three squirts of milk were discarded, and a sample of approximately five ml milk was taken into the corresponding tube, starting with the nearest quarter. The tube was held as horizontally and as far away from the teat as possible to avoid contamination.

The milk and teat skin samples were transported on ice and kept refrigerated at 4°C until next morning where the laboratory work was initiated. Plating of milk and teat skin samples was done within 24 hours of sampling.

Bacteriological culture

All laboratory work was carried out at Department of Bacteriology & Parasitology, DTU Vet, National Veterinary Institute, Frederiksberg, Denmark.

Milk samples: After acclimatization to room tempearture, tubes were vortexed five to 10 seconds, and 10 µl were streaked with a disposable calibrated 10 µl loop aseptically onto a quarter of both a calf blood agar (prepared at DTU Vet laboratory), a SA*Select*™ medium (Bio-Rad), and a modified Edward's medium (Oxoid, supplemented with 5 % calf blood and 2 % filtrate of a b-toxin producing *S. aureus*, Appendix I). The plates were marked with sample ID and quarter for identification.

Teat skin samples: After acclimatization to room temperature, tubes were vortexed for 20 seconds, and the swabs were removed with a sterile tweezer, sterilized in flames. With pipette, 100 µl were inoculated and spread with a steel drigalski spatula on a whole calf blood agar, SA*Select*™ medium, and modified Edward's medium. Plates were marked with sample ID and quarter for identification.

The plates were incubated aerobically at 37 °C for 48 hours in total and read after 24 hours and again after 48 hours. The approximate number of CFU was determined by colony counting. If the number of CFU was between 1 and 100, every colony was counted, if the number of CFU was above 100, the approximate number of CFU in 100 colony steps was estimated.

After plating, the milk and teat skin samples were stored at -18°C.

Identification of bacteria

S. agalactiae: On modified Edward's medium, suspected colonies were small, white to grey and with CAMP reaction. On calf blood agar, suspected colonies were small to medium, white to grey, and had a clear zone of hemolysis. Suspected colonies were subcultured on calf blood agar for confirmation. Isolates were confirmed as *S. agalactiae* using latex agglutination for Lancefield group B (PathoDxtra™ Strep Grouping Kit) or MALDI-TOF MS. MALDI-TOF MS was performed by trained technicians at DTU Vet.

S. aureus: On SA*Select*™ medium, suspected colonies were small and pink or orange. On calf blood agar, suspected colonies were creamy and golden yellow with a double hemolysis. Suspected colonies were subcultured on calf blood agar for confirmation. Isolates were confirmed as *S. aureus* using MALDI-TOF MS.

A quarter was defined as positive in milk with BC if at least one colony (CFU ≥ 1) of *S. aureus* or *S. agalactiae* appeared on any of the used agar plates. Likewise, a quarter was defined as positive on teat skin with BC if at least one colony (CFU ≥ 1) of *S. aureus* or *S. agalactiae* appeared on any of the used agar plates.

Mastit4 qPCR Assay

For every RH quarter, two PCR swabs (DNA Diagnostic FLOQSwabs) were immersed in the corresponding milk and teat skin sample, respectively, immediately after plating. The PCR swabs were sent by mail to DNA Diagnostic A/S where the analysis was done in their development laboratory (see laboratory protocol in Appendix II). The Mastit4 qPCR Assay includes several kits (Mastit4BDF), all in all 12 different pathogens were analyzed, including *S. agalactiae, S. aureus, Mycoplasma bovis, Streptococcus uberis* (*S. uberis*), **β**-Lactamase from staphylococci, CNS, *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Mycoplasma spp., Klebsiella, Enterococcus, Lactococcus lactis spp. lactis, E. coli*, and *Prototheca*. A quarter was defined as positive in either milk or on teat skin if the Ct value for either *S. aureus* or *S. agalactiae* was < 40.

The approximate volume soaked by the PCR swab was 220 µl, which after DNA extraction and purification steps leaves approximately 10 µl of the original sample for qPCR analysis, which in concentration per ml fairly corresponds to the 10 µl inoculated on plates for milk samples (Katholm 2017, personal communication). For teat skin samples, 100 µl were inoculated on plates why the qPCR analysis was done on a 10 times smaller sample volume.

Part B

Herd selection

From March 2nd to April 25th, 2017, each herd from part A was revisited with the purpose of resampling quarters tested positive in part A for either *S. agalactiae* or *S. aureus* on either milk or teat skin with either BC or PCR. The time interval between part A and part B varied between herds, from seven to 22 days (Table 1).

Herd	Part A (date)	Part B (date)	Interval (days)
H1	14.02.2017	02.03.2017	16
H2	20.02.2017	02.03.2017	10
H3	06.03.2017	23.03.2017	17
H4	13.03.2017	23.03.2017	10
H5	20.03.2017	30.03.2017	10
H6	27.03.2017	03.04.2017	7
H7	03.04.2017	25.04.2017	22
H8	18.04.2017	25.04.2017	7

Table 1 Dates for the two rounds of herd visits (part A and B) and time interval (days) in herd (H) 1 to 8.

Cow and quarter selection

Based on the results from BC and PCR from part A, where positive was defined as Ct < 40 for PCR and CFU ≥ 1 for BC, cows and quarters for part B were selected. A maximum of 20 quarters per herd and one quarter per cow were selected with following prioritized criteria: 1) teat skin samples positive for *S. agalactiae* with PCR or BC, 2) milk samples positive for *S. agalactiae* with PCR or BC, 3) teat skin samples positive for *S. aureus* with PCR or BC, and 4) milk samples positive for *S. aureus* with PCR or BC. If more quarters were positive with same criteria fulfilled, RH or hind quarters were selected if possible. If more positive criteria per quarter were fulfilled, e.g. if a quarter was positive with both bacteria or both methods (PCR and BC), this quarter was preferred over the RH quarter.

Sample collection

Teat skin samples: Due to different aims, the sampling procedure for teat skin samples in part B differed from the sampling procedure for teat skin samples in part A. Whereas the aim of part A (regarding teat skin samples) was to compare the sensitivity and specificity of the wet-dry method with the PCR swab immersed into the same Ringer's solution, the aim of part B was to compare the sensitivity and specificity of the wet-dry method with the PCR swab rolled directly on teat skin. Therefore, the sampling procedure for teat skin samples included swabbing with both the PCR swab and the wet-dry swabs for BC.

On beforehand, to prevent bias, the sequence of swabs was selected so that in around half of the samples, the wet-dry swabs for BC were sampled prior to the PCR swab, and in the other half of the samples, the PCR swab was sampled prior to the wet-dry swabs for BC.

As in part A, a new pair of gloves was worn for each cow. The selected teat was cleaned with at least one dry paper towel. Prior to sampling, the PCR swab for teat skin analysis was moistened

in a buffer solution from DNA Diagnostic A/S. Then, the PCR swab was rolled 360° around the teat canal orifice at a distance of one cm and returned to its tube. The wet-dry swabs for BC followed the wet-dry method described by Paduch & Krömker (2011) as in part A.

Milk samples: A sterile milk sample was collected as described in part A. The PCR swab for milk sample analysis was shortly immersed into the fresh milk sample until completely covered with milk, immediately after sampling (thus before plating opposite to part A).

The milk and teat skin samples were transported on ice and kept refrigerated at 4 °C until next morning where the laboratory work was initiated. Plating of milk and teat skin samples was done within 24 hours of sampling.

Bacteriological culture

Milk samples: After acclimatization to room temperature, tubes were vortexed five to 10 seconds, and 10 µl were streaked with a disposable calibrated 10 µl loop aseptically onto a quarter of both a calf blood agar and a modified Edward's medium. The plates were marked with sample ID for identification.

Teat skin samples: After acclimatization to room temperature, tubes were vortexed for 20 seconds, and the swabs were removed with a sterile tweezer, sterilized in flames. With pipette, 100 µl were inoculated and spread with a steel drigalski spatula on a whole calf blood agar and modified Edward's medium. Plates were marked with sample ID for identification.

The plates were incubated aerobically at 37 °C and read after 48 hours of incubation.

Identification of bacteria

The identification and confirmation of bacteria followed the same criteria as in part A. The approximate number of CFU was determined by colony counting.

A quarter was defined as positive in milk with BC if at least one colony (CFU ≥ 1) of *S. aureus* or *S. agalactiae* appeared on any of the used agar plates. Likewise, a quarter was defined as positive on teat skin with BC if at least one colony (CFU ≥ 1) of *S. aureus* or *S. agalactiae* appeared on any of the used agar plates.

Mastit4 qPCR Assay

As stated, the PCR swab for teat skin analysis was moistened in a buffer solution and rolled directly on the teat skin. The PCR swab for milk sample analysis was immersed into the fresh milk sample

at the herd visit immediately after sampling. The PCR swabs were sent by mail to DNA Diagnostic A/S where the analysis was done.

Bacteriological culture: Colony counting

To increase the sensitivity of BC for the detection of *S. aureus* and *S. agalactiae*, both calf blood agar plates and a selective medium (SA*Select*™ medium and modified Edward's medium for *S. aureus* and *S. agalactiae*, respectively) were used, as already described. For this reason, estimation of the number of CFU for both *S. aureus* and *S. agalactiae* was carried out on both calf blood agar and the selective medium. Analyses were primarily based on the number of CFU counted on the selective medium. In cases where the selective medium was negative (or missing) and the calf blood agar was positive, the number of CFU from the calf blood agar was used. The agreement between the calf blood agar and the selective medium for the detection of *S. aureus* and *S. agalactiae* in both part A and B is seen in Appendix III.

Supplemental tests

Inoculum amount teat skin samples part B

To investigate whether the sensitivity of BC could be increased, different inoculum amounts were tested for samples from herd 1 and 2 in part B. From teat skin samples, 100 and 200 µl were inoculated on modified Edward's medium and calf blood agar, and from milk samples, 10 and 100 µl were inoculated.

Todd Hewitt broth

Sixteen teat skin samples from herd 1 to 3 positive for *S. agalactiae* with PCR but negative with BC in part A (kept in the refrigerator for several weeks) and all teat skin samples from herd 7 and 8 in part B (at the same time as plating) were recultured after enrichment step with Todd Hewitt broth containing colistin and nalidixid acid (LIM Broth, Thermo Scientific, Denmark).

Teat skin samples were vortexed for 10 seconds, and 0.5 ml were transferred into separate glasses of Todd Hewitt broth with pipette. Tubes were incubated for 24 hours with loosened caps. 0.1 ml of the broth were streaked on both calf blood agar and modified Edward's medium. The plates were read after 24 and again after 48 hours.

Statistical analyses part A and B

The data was recorded with Microsoft Excel 2016 software (Microsoft, USA). Prior to statistical analyses, the plausibility and the completeness of data were verified, and the bacterial counts were categorized into four CFU groups based on 10-log transformation.

SAS software (University Edition, Copyright ® 2012 – 2016, SAS Institute Inc., Cary, NC, USA) was used for the following analyses. Descriptive statistics (median, mean, standard deviation, minimum, maximum, quartiles) on Ct values from PCR results from part A and part B was generated using PROC MEANS. Box-and-whisker plots showing associations between Ct values and CFU groups for *S. agalactiae* and *S. aureus* from both milk and teat skin samples were generated using PROC SGPLOT. To analyze the association between Ct values and CFU groups, both variables on ordinal scale, the Spearmen Rank Correlation Coefficient was calculated using PROC CORR. Results were defined as significant if $P < 0.05$.

To analyze the agreement between PCR and BC, kappa and corresponding 95 % confidence intervals (CI) were calculated using VassarStats (Vassar College, Poughkeepsie, New York, USA). To evaluate equal probabilities for being tested positive with the two methods (Ersbøll et al. 2004), McNemar statistic values (χ^2) and p-values were calculated using the Excel sheet '2by2' (Toft 2001, Copenhagen University). The critical value was 3.85, and statistical significance was defined as $P \leq 0.05$. Different Ct value cutoffs for PCR were chosen ($Ct < 40, \leq 37, \leq 32$) for the calculation of both kappa and McNemar statistic values. Cutoff ≤ 37 was chosen based on recommendations from the manufacturer (Katholm 2017, personal communication) whereas cutoff ≤ 32 was based on the visual distribution of Ct values and according to the results from Bennedsgaard et al. (2016).

The traditional method to evaluate the performance of a diagnostic test by calculating sensitivity and specificity demands a reference standard. The reference standard will classify animals as diseased or disease-free. Based on this knowledge, the sensitivity of the test (the true positive fraction), the specificity (the true negative fraction), the positive predictive value (PPV) (the proportion of positive truly positive), and the negative predictive value (NPV) (the proportion of negative truly negative) can be calculated (Toft et al. 2005).

In the absence of a reference standard to classify true cases of IMI and teat skin colonization with *S. agalactiae* and *S. aureus*, the test characteristics (sensitivity and specificity) of PCR and BC were estimated using a Bayesian latent class analysis (LCA) model described by Hui & Walter (1980) with the following assumptions: 1) the population is divided into two or more populations

(with different disease prevalence) in which two or more tests are evaluated, 2) the sensitivity and specificity of the tests are the same in all populations, and 3) the tests are conditionally independent given the disease status. Sensitivity and specificity estimates of the two tests and the prevalence in each population were calculated.

Eight herds were divided into two populations based on AMS type (Lely or DeLaval). It was assumed a priori that the prevalence of *S. agalactiae* and *S. aureus* in the milk and teat skin samples from the two populations would differ, that the test characteristics of PCR and BC could be regarded as constant across populations, and that conditional independence between PCR and BC existed given the infection status (since PCR is built on detection of amplified target DNA whereas BC is built on isolation and identification of viable bacterial cells).

The model was implemented in the freeware program OpenBUGS, version 3.2.3 rev. 1012 (Thomas et al. 2006). OpenBUGS uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution. The first 10.000 MC samples were discarded as a burn-in to allow convergence, and the following 20.000 iterations were used for posterior inference. Convergence of the MCMC chain after initial burn-in was assessed by visual inspection of the time-series plots. Posterior inference was done by calculating mean, standard deviation, median, and 95 % posterior credibility intervals (PCI) of the prevalence in the two populations and the sensitivity and specificity of the two tests. Analyses on milk samples for both *S. agalactiae* and *S. aureus* were done using Ct value cutoff ≤ 37 for PCR, based on recommendations from the manufacturer (Katholm 2017, personal communication; Timonen et al. 2017). Due to few or zero positive teat skin samples for both *S. agalactiae* and *S. aureus* together with the demand for division of the herds into two populations with different disease prevalence, no test characteristics could be estimated using LCA.

Since LCA was not applicable to teat skin samples for *S. aureus* and *S. agalactiae*, sensitivity and specificity, PPV, and NPV were calculated using BC as a reference standard at Ct value cutoff ≤ 37. When compared with BC, the relative sensitivity is defined as the proportion of true positive results (according to BC) correctly identified with PCR. Likewise, the relative specificity is defined as the proportion of true negative results (according to BC) correctly identified with PCR (Steele et al. 2017). Sensitivity and specificity were calculated separately for *S. agalactiae* and *S. aureus*. This calculation was done using Vassarstats.net (2017), calculating 95 % CI corrected for continuity according to the efficient-score method described by Newcombe (1998).

Results

Herd	Herd size (cows)	AMS type (number of units)
H1	250	Lely (4)
H2	220	Lely (3)
H3	360	Lely (7)
H4	300	Lely (5)
H5	220	Lely (4)
H6	250	Lely (4)
H7	350	DeLaval (6)
H8	250	DeLaval (4)

Table 2 Herd size (cows) and AMS type (Lely or DeLaval) for sampled herds (H). Number of units is shown in brackets. Data collected from the Danish 'CHR-register'.

Figure 1 Map of Denmark showing location of sampled herds. Yellow dots represent herds with DeLaval robots, red dots represent herds with Lely robots. Map created with inspiration from http://www.videnscenterfordemens.dk/statistik/forekomst-af-demens-i-danmark/

Part A

From February 14th to April 18th, 2017, milk and teat skin samples from all four quarters from 306 cows were collected. Location of and information about sampled herds are given in Figure 1 and Table 2, respectively. From among these 306 cows, 19 RH quarters were excluded because the quarters were dry (13), missing the teat (1), or sampled twice by mistake (5). Thus 287 RH quarters were included for PCR analysis and subsequent comparison between PCR and BC.

Distribution of positive samples

BC

The distribution of samples positive with BC (CFU ≥ 1) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin is shown for each herd in Table 3. While *S. aureus* was detected in 23 teat skin samples, ranging from one to eight teat skin samples within positive herds, *S. agalactiae* could only be cultured from the teat skin in one herd. In two herds, *S. aureus* was not detected on teat skin. Both *S. agalactiae* and *S. aureus* were not isolated from milk samples in two (different) herds. *S. agalactiae* was detected in 19 milk samples, ranging from one to six milk samples within positive herds, and *S. aureus* was detected in 22 milk samples, ranging from one to 11 milk samples within positive herds. *S. agalactiae* was not isolated from milk or teat skin in herd 2 and 6 whereas *S. aureus* was not isolated from milk or teat skin in herd 4.

Bacteriological culture	Milk *S. agalactiae* (N)	Milk *S. aureus* (N)	Teat *S. agalactiae* (N)	Teat *S. aureus* (N)
H1 (28 q)	1	5	0	0
H2 (36 q)	0	1	0	1
H3 (38 q)	3	0	0	5
H4 (35 q)	3	0	1	0
H5 (37 q)	3	1	0	4
H6 (40 q)	0	1	0	3
H7 (34 q)	6	3	0	8
H8 (39 q)	3	11	0	2
Total (287 q)	19	22	1	23

Table 3 Samples positive with bacteriological culture (BC) (colony forming units (CFU) ≥ 1) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin in relation to herd. The number of quarters (q) sampled at each herd (H) is shown in brackets.

PCR

The distribution of samples positive with PCR with either *S. aureus* or *S. agalactiae* in either milk or on teat skin is shown for each herd in Table 4. The distribution is shown for both Ct value cutoff ≤ 37 and ≤ 32. Since only the number of positive samples with *S. aureus* on teat skin differed between Ct value cutoff < 40 and ≤ 37 (lowering the cutoff from < 40 to ≤ 37 reduced the total number of positive samples from 45 to 42), this cutoff has not been included in the table.

S. agalactiae was detected on teat skin in all herds independently of Ct value cutoff. At Ct value cutoff ≤ 37, the number of positive samples ranged from one to 16. Unlike *S. agalactiae*, the detection of *S. aureus* on teat skin depended on Ct value cutoff. At Ct value cutoff ≤ 37, *S. aureus* was detected in seven out of eight herds, ranging from one to 16 positive teat skin samples. At Ct value cutoff ≤ 32, *S. aureus* was detected in only three out of eight herds.

In only one herd (herd 2), *S. agalactiae* was not detected in milk samples. In the other herds, the number of positive samples ranged from one to 18 at Ct value cutoff ≤ 37. In only one herd (herd 4), *S. aureus* was not detected in milk samples. In the other herds, the number of positive samples ranged from one to 14 at Ct value cutoff ≤ 37. *S. aureus* was neither detected in milk nor on teat skin in herd 4.

Lowering the Ct value cutoff from ≤ 37 to ≤ 32 did not have a great impact on the total number of positive samples with *S. agalactiae* and *S. aureus* in milk samples, indicating that the majority of positive samples was below Ct value cutoff ≤ 32. On the other hand, lowering the Ct value cutoff from ≤ 37 to ≤ 32 had greater impact on the number of positive teat skin samples with especially *S. aureus* (from 42 to 15 positive teat skin samples).

PCR	Milk *S. agalactiae* (N)		Milk *S. aureus* (N)		Teat *S. agalactiae* (N)		Teat *S. aureus* (N)	
Ct cutoff	≤ 37	≤ 32	≤ 37	≤ 32	≤ 37	≤ 32	≤ 37	≤ 32
H1 (28 q)	1	1	6	5	1	1	5	3
H2 (36 q)	0	0	2	1	11	7	16	6
H3 (38 q)	5	5	1	1	3	3	5	0
H4 (35 q)	18	16	0	0	16	16	0	0
H5 (37 q)	3	3	2	2	4	3	3	0
H6 (40 q)	3	3	1	1	14	9	11	6
H7 (34 q)	7	6	3	3	2	1	1	0
H8 (39 q)	3	3	14	11	1	1	1	0
Total (287 q)	40	37	29	24	52	41	42	15

Table 4 Samples positive with polymerase chain reaction (PCR) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin in relation to herd. The distribution is shown for both cycle threshold (Ct) value cutoff ≤ 37 and ≤ 32. The number of quarters (q) sampled at each herd (H) is shown in brackets.

Distribution of Ct values

The frequency distribution of Ct values in relation to pathogen (*S. aureus* or *S. agalactiae*) and sample type (milk or teat skin) is shown in Figure 2, and the descriptive statistics are shown in Table 5 (Ct value 40 not shown).

In general, at Ct value cutoff < 40, *S. agalactiae* and *S. aureus* were detected in more teat skin samples (52 and 45, respectively) than milk samples (40 and 29, respectively), and the Ct values for teat skin samples were higher than those for milk samples. Furthermore, the Ct values for *S. aureus* on teat skin were generally higher than those for *S. agalactiae* on teat skin (Figure 2). This was also supported by the difference in median for *S. aureus* and *S. agalactiae* on teat skin (Table 5). The range of Ct values for milk samples was wider than for teat skin samples which can be seen in both Figure 2 and Table 5.

	N	Mean	Median	Std Dev	Min.	Max.	25th perc.	75th perc.
***S. agalactiae* milk**	40	22.1	21.6	6.9	9.8	37.0	16.5	28.0
***S. aureus* milk**	29	22.4	22.4	7.9	12.8	36.7	14.8	28.3
***S. agalactiae* teat**	52	29.7	30.1	3.1	22.0	36.8	27.6	31.8
***S. aureus* teat**	45	33.2	33.4	2.7	26.6	39.9	31.1	34.4

Table 5 Descriptive statistics of the cycle threshold (Ct) values, including number (N), mean, median, standard deviation (Std Dev), minimum value (Min.), maximum value (Max.), 25th percentile (25th perc.) and 75th percentile (75th perc.). Ct value 40 is not included. In total, milk and teat skin samples were collected from 287 quarters.

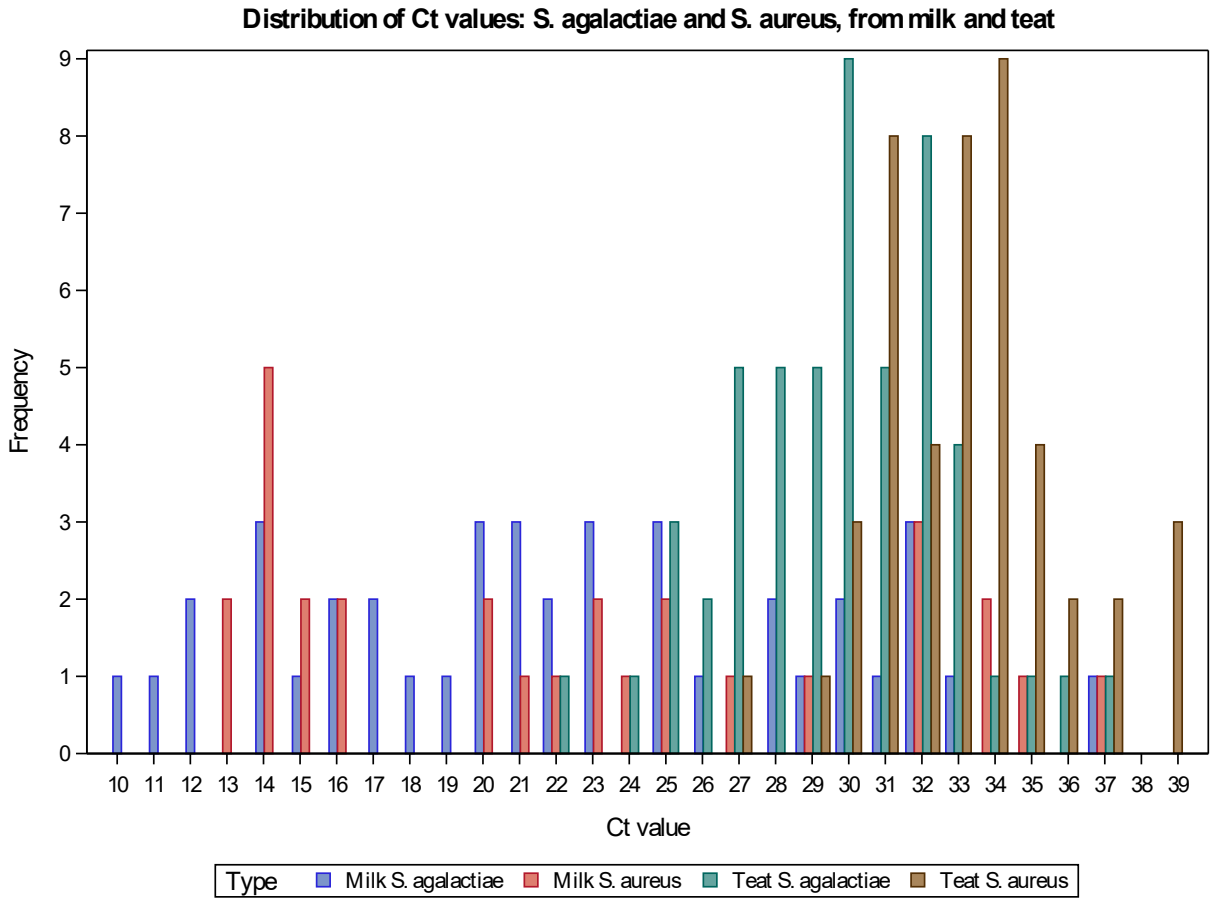


Figure 2 The distribution of cycle threshold (Ct) values in relation to pathogen (*S. aureus* or *S. agalactiae*) and sample type (milk or teat skin). Ct value 40 is not included. In total, milk and teat skin samples were collected from 287 quarters.

Distribution of Ct values in relation to CFU group

In the following, the association between Ct values and the number of CFU was investigated. For this reason, the number of CFU was grouped by number where CFU group 0, 1, 2, and 3 referred to zero, between one and 10, between 11 and 100, and between 101 and 1000 CFU, respectively. For milk samples, the number of CFU was stated per 0.01 ml milk whereas for teat skin samples, the number of CFU was stated per 0.1 ml of Ringer's solution.

S. agalactiae in milk samples

In Figure 3, the distribution of Ct values in relation to CFU group is shown for *S. agalactiae* in milk samples. There might have been an association between the Ct values and the CFU group. The Spearman Rank Correlation Coefficient of -0.7 [-0.75;-0.63] showed a strong and significant ($p < 0.0001$) negative relation between the two variables, implying that Ct values decreased with increasing CFU group. Twenty-one samples (Table 11 at Ct value cutoff ≤ 37) were positive with PCR but negative with BC. The Ct values in BC negative samples ranged from 14 to 37 (Figure

3). Note that the CFU group 1 contained only two observations whereas the remaining 17 BC positive samples were evenly distributed between CFU group 2 and 3.

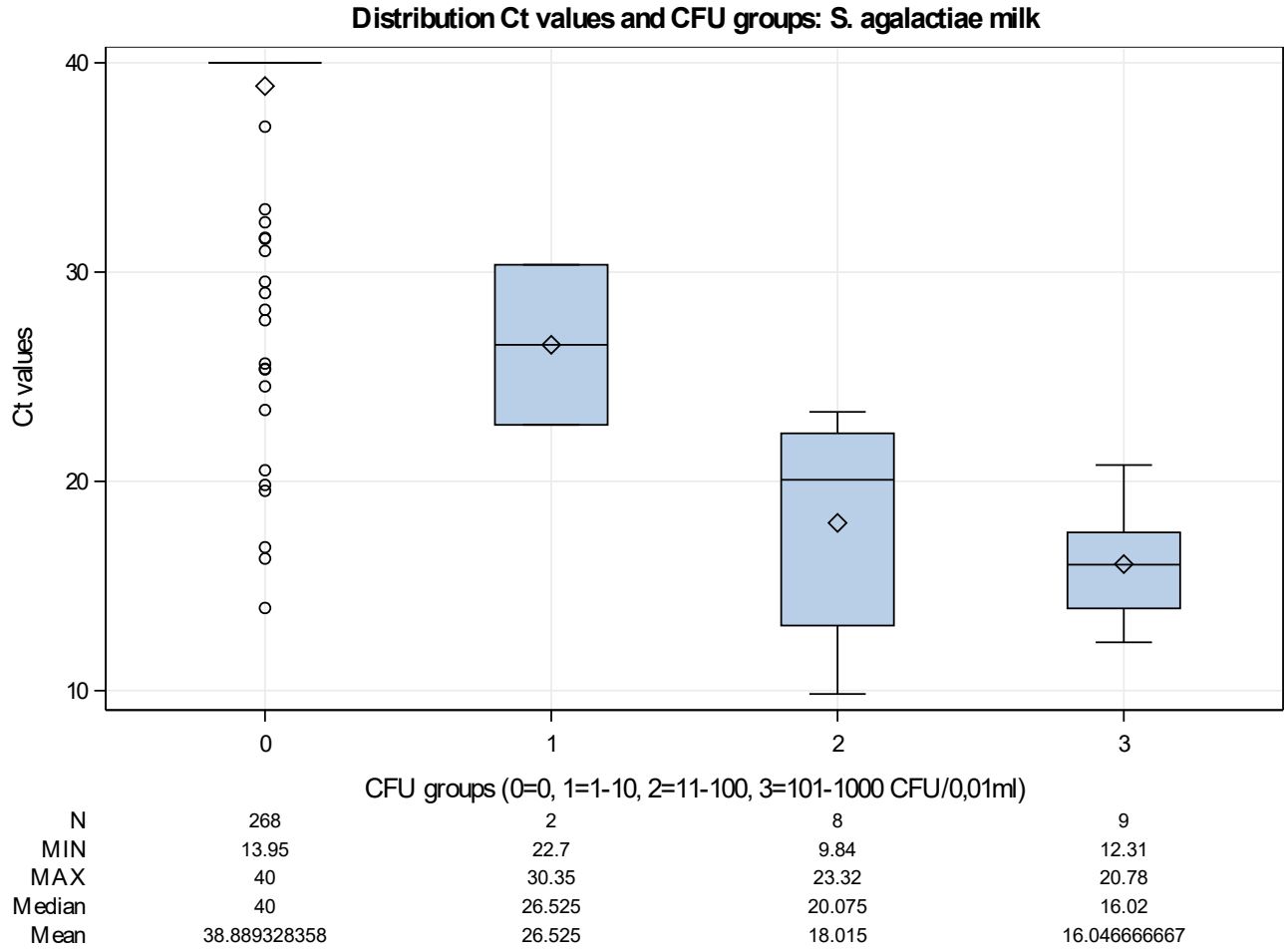
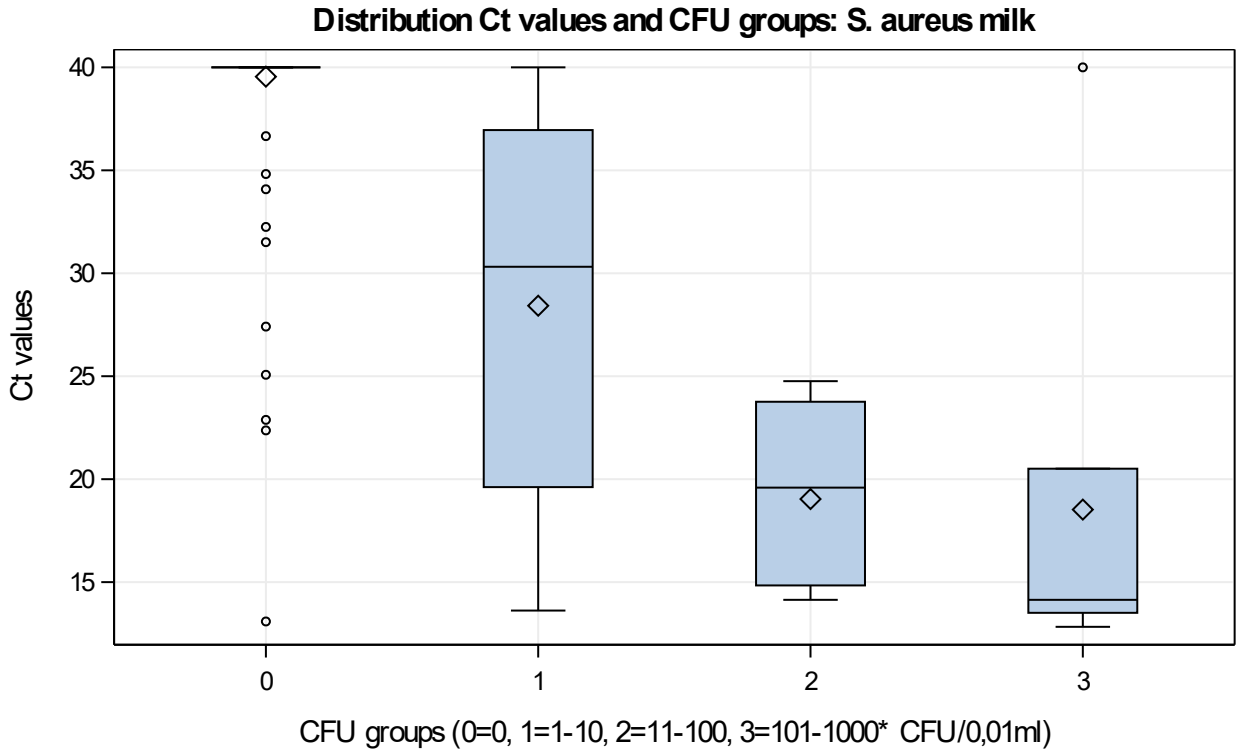


Figure 3 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. agalactiae* in milk samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean.

S. aureus in milk samples

In Figure 4, the distribution of Ct values in relation to CFU group is shown for *S. aureus* in milk samples. This figure indicated an association between the Ct values and the CFU group. The Spearman Rank Correlation Coefficient of -0.75 [-0.79;-0.69] showed a strong and significant ($p < 0.0001$) negative relation between the two variables, implying that Ct values decreased with increasing CFU group. Ten samples (Table 11 at Ct value cutoff ≤ 37) were positive with PCR but negative with BC. The Ct values in BC negative samples ranged from 13 to 37 (Figure 4). The 22 BC positive samples were evenly distributed among CFU group 1, 2, and 3. Three samples (Table 11 at Ct value cutoff ≤ 37) were positive with BC but negative with PCR of which one had above 100 CFU.



	0	1	2	3
N	265	8	7	7
MIN	13.09	13.62	14.14	12.83
MAX	40	40	24.76	40
Median	40	30.315	19.59	14.14
Mean	39.547698113	28.42375	19.035714286	18.522857143

Figure 4 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. aureus* in milk samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean. *CFU group 3 contained one observation with above 1000 CFU/0.01 ml (with a corresponding Ct value of 14).

S. agalactiae in teat skin samples

Whereas 52 teat skin samples had a positive PCR test for *S. agalactiae*, only one quarter was positive with BC (Table 11 at Ct value cutoff ≤ 37) which is why no analysis based on the graphical illustration of the association between Ct values and CFU group was made. Fifty-one samples were positive with PCR but negative with BC. The distribution of Ct values in BC negative samples is seen in Figure A in Appendix IV.

S. aureus in teat skin samples

In Figure 5, the distribution of Ct values in relation to CFU group is shown for *S. aureus* in teat skin samples. Since there was only one CFU group (CFU group 1) besides CFU group 0, the foundation for concluding an association between Ct values and CFU group was insufficient. This was supported by the Spearman Rank Correlation Coefficient of -0.13 [-0.24;-0.01] which implied a very weak, yet significant (p = 0.0278), negative relation between the Ct values and the CFU

group. As it is apparent from Table 11 and illustrated by Figure 5, sixteen samples (at Ct value cutoff ≤ 37) were positive with BC but negative with PCR. The number of CFU for PCR negative samples was low, ranging from one to 10. Thirty-five samples were positive with PCR but negative with BC (Table 11 at Ct value cutoff ≤ 37) but the Ct values were high.

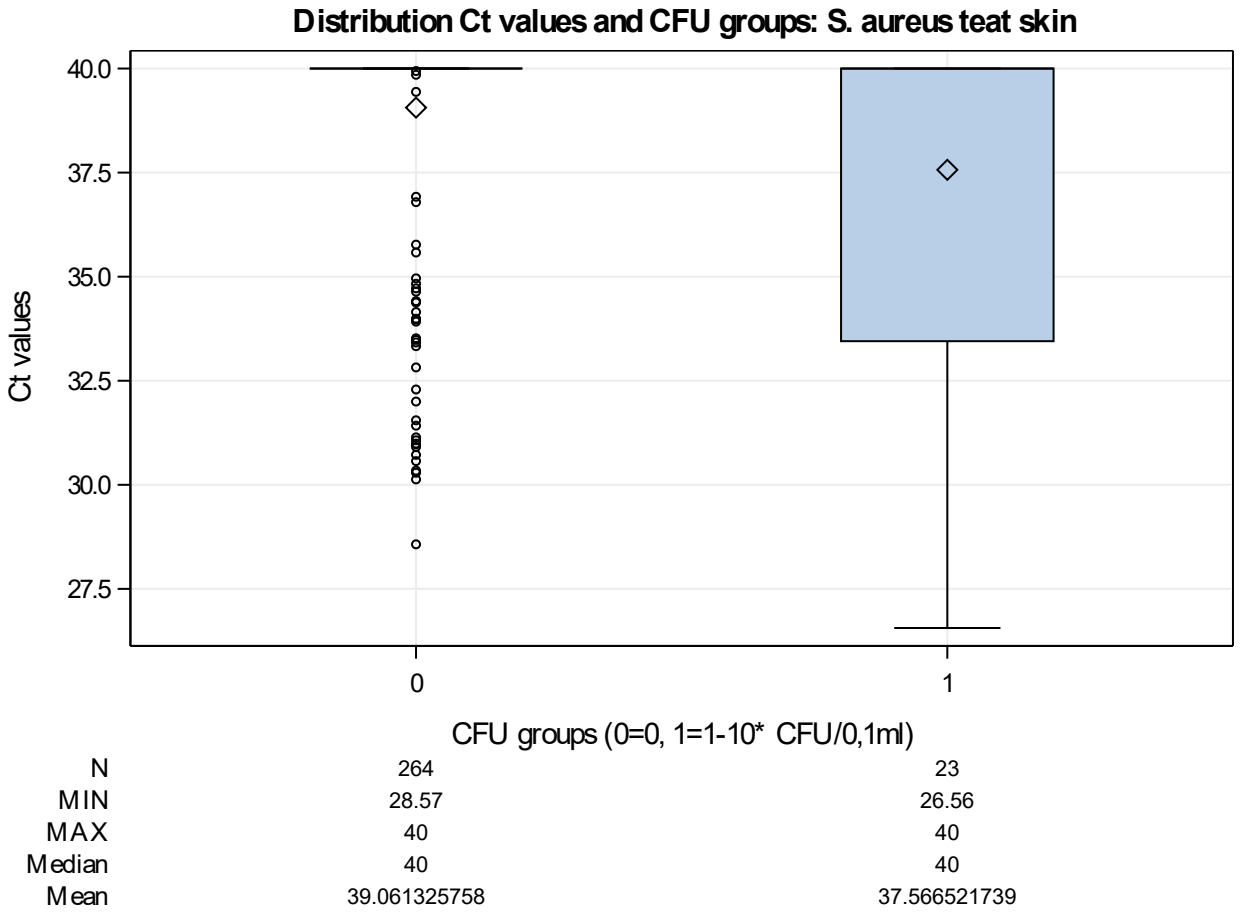


Figure 5 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. aureus* in teat skin samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean. *CFU group 1 contained one observation with 12 CFU/0.1 ml (with a corresponding Ct value of 32).

Part B

From March 2nd to April 25th, 2017, each of the eight herds was revisited. On beforehand, 159 quarters, all from different cows, were selected based on the results from PCR and BC from part A. Twenty-seven cows were dried off, dead, or culled since part A, or simply not sampled in part B by mistake, and thus excluded, resulting in milk and teat skin samples from 132 (not necessarily RH) quarters.

Sampling procedure

As described in materials and methods, the teat skin samples in part B were collected with alternate sampling procedures. Appendix V lists all positive and negative samples in relation to the sampling procedure. In 64 out of 132 samples (48 %), the wet-dry swabs for BC were sampled prior to the PCR swab (sampling procedure: BC first). In 68 out of 132 samples (52 %), the PCR swab was sampled prior to the wet-dry swabs for BC (sampling procedure: PCR first). The distributions of Ct values for the two sampling procedures (BC first and PCR first) were compared for both *S. agalactiae* and *S. aureus*. As demonstrated in Figure 6 and 7, there was no difference in the Ct value distribution for the two sampling procedures. Hence, the teat skin samples were used for further analyses without adjusting for the sequence of the two methods.

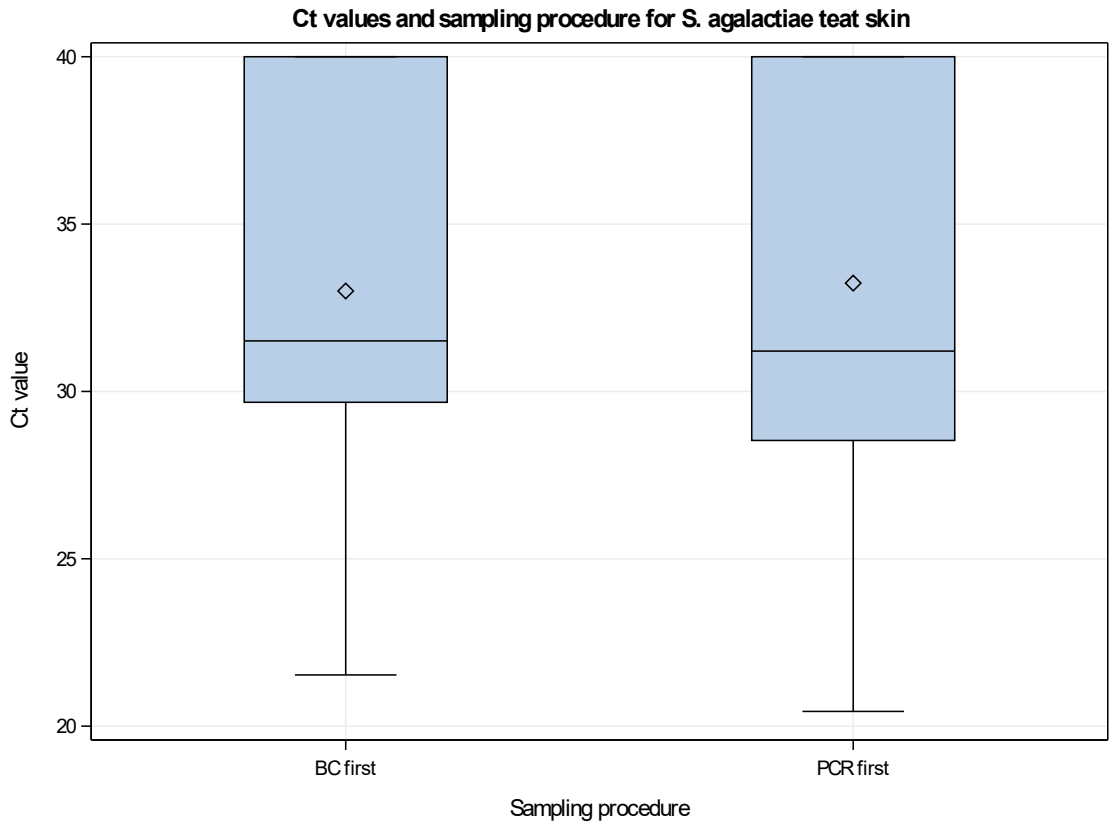


Figure 6 Distribution of cycle threshold (Ct) values in relation to sampling procedure (wet-dry swabs for bacteriological culture (BC) first (BC first) or swab for polymerase chain reaction (PCR) first (PCR first)) for *S. agalactiae* on teat skin.

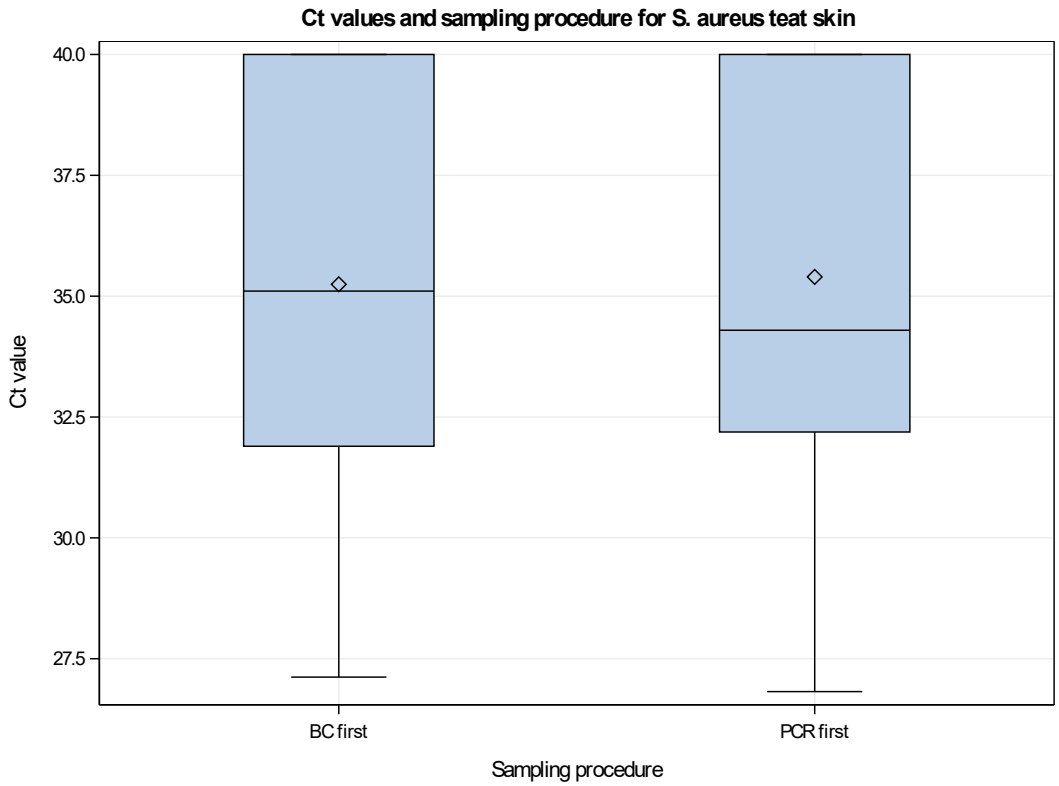


Figure 7 Distribution of cycle threshold (Ct) values in relation to sampling procedure (wet-dry swabs for bacteriological culture (BC) first (BC first) or swab for polymerase chain reaction (PCR) first (PCR first)) for *S. aureus* on teat skin.

Distribution of positive samples

BC

The distribution of samples positive with BC (CFU ≥ 1) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin is shown for each herd in Table 6. Since the quarters were selected based on results from part A, we cannot comment on the prevalence but just point out that once again we isolated *S. agalactiae* from only one teat skin sample (neither from the same quarter nor the same herd).

Bacteriological culture	Milk *S. agalactiae* (N)	Milk *S. aureus* (N)	Teat *S. agalactiae* (N)	Teat *S. aureus* (N)
H1 (10 q)	1	2	0	0
H2 (11 q)	0	0	0	0
H3 (17 q)	4	0	0	0
H4 (19 q)	2	0	0	0
H5 (20 q)	5	0	0	3
H6 (20 q)	2	0	0	2
H7 (17 q)	8	1	0	1
H8 (18 q)	7	7	1	5
Total (132 q)	29	10	1	11

Table 6 Samples positive with bacteriological culture (BC) (colony forming units (CFU) ≥ 1) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin in relation to herd. The number of quarters (q) sampled at each herd (H) is shown in brackets.

PCR

Table 7 shows the frequency of positive PCR samples with either *S. aureus* or *S. agalactiae* in either milk or on teat skin at different Ct value cutoffs (≤ 37 and ≤ 32). Looking at the differences from Ct value cutoff < 40 and ≤ 37 (data not shown), *S. aureus* in milk and on teat skin was the only pathogen with changes in the total number of positive samples (milk samples from 24 to 22 positive samples and teat skin samples from 85 to 80 positive samples), therefore Ct value cutoff < 40 was not included in the table. Once again, due to the selection criteria, we cannot comment on the prevalence. Lowering the Ct value cutoff from ≤ 37 to ≤ 32 especially impacted the total number of positive teat skin samples with *S. aureus*, reducing the total number from 80 to 31 positive teat skin samples.

PCR	Milk *S. agalactiae* (N)		Milk *S. aureus* (N)		Teat *S. agalactiae* (N)		Teat *S. aureus* (N)	
Ct cutoff	≤ 37	≤ 32	≤ 37	≤ 32	≤ 37	≤ 32	≤ 37	≤ 32
H1 (10 q)	1	1	3	3	2	2	7	1
H2 (11 q)	1	1	1	0	11	11	5	2
H3 (17 q)	5	5	2	0	7	7	5	1
H4 (19 q)	6	6	0	0	7	6	4	1
H5 (20 q)	6	6	2	2	14	12	17	11
H6 (20 q)	2	1	0	0	18	18	16	7
H7 (17 q)	9	9	3	2	13	10	12	0
H8 (18 q)	8	7	11	10	13	11	14	8
Total (132 q)	38	36	22	17	85	77	80	31

Table 7 Samples positive with polymerase chain reaction (PCR) with either *S. aureus* or *S. agalactiae* in either milk or on teat skin in relation to herd. The distribution is shown for both cycle threshold (Ct) value cutoff ≤ 37 and ≤ 32. The number of quarters (q) sampled at each herd (H) is shown in brackets.

Quarters positive in both part A and B

From among the 132 quarters sampled in part B, 87 were RH quarters and thus analyzed with PCR in both part A and B. This enabled the comparison of quarters positive with PCR (Ct value cutoff < 40) in both parts of the study (Table 8) and the comparison of quarters positive with BC (CFU ≥ 1) in both parts of the study (Table 9). Especially the findings from the PCR positive teat skin samples were interesting. Forty-nine of the selected teat skin samples were positive with *S. agalactiae* in part A of which 37 (77 %) were still positive with *S. agalactiae* in part B.

PCR	Pathogen	Positive, part A N	Positive, part B N (%)
Milk	*S. agalactiae*	26	18 (70)
	S. aureus	12	8 (67)
Teat	*S. agalactiae*	49	37 (77)
	S. aureus	26	21 (80)

Table 8 Comparison of quarters positive with polymerase chain reaction (PCR) (cycle threshold (Ct) value cutoff < 40) in both part A and B. The percentage of quarters positive in both parts of the study is shown in brackets. In total, 87 right hind (RH) quarters were analysed twice with PCR between 7 and 22 days apart.

BC	Pathogen	Positive, part A N	Positive, part B N (%)
Milk	*S. agalactiae*	30	27 (90)
	S. aureus	21	10 (48)
Teat	*S. agalactiae*	2	0
	S. aureus	34	8 (24)

Table 9 Comparison of quarters positive with bacteriological culture (BC) (colony forming units (CFU) ≥ 1) in both part A and B. The percentage of quarters positive in both parts of the study is shown in brackets. In total, 132 quarters were analysed twice with BC between 7 and 22 days apart.

Distribution of Ct values

The frequency distribution of Ct values in relation to pathogen (*S. aureus* or *S. agalactiae*) and sample type (milk or teat skin) is shown in Figure 8, and the descriptive statistics are shown in Table 10. For better overview, Ct value 40 was excluded.

The same pattern as in part A was seen for part B: Ct values for teat skin samples were higher than those for milk samples. Furthermore, the Ct values for *S. aureus* on teat skin were generally higher than those for *S. agalactiae* on teat skin (Figure 8). This was also supported by the difference in median for *S. aureus* and *S. agalactiae* on teat skin (Table 10). The range of Ct values for milk samples was wider than for teat skin samples which can be seen in both Figure 8 and Table 10. The median for *S. aureus* in milk samples was higher in part B (median = 26.3) than in part A (median = 22.4).

	N	Mean	Median	Std Dev	Min.	Max.	25th perc.	75th perc.
***S. agalactiae* milk**	38	22.5	21.8	5.2	10.9	32.3	18.9	25.7
***S. aureus* milk**	24	25.5	26.3	9.0	13.5	38.8	16.1	34.5
***S. agalactiae* teat**	85	29.3	29.9	2.7	20.4	34.5	28.0	31.2
***S. aureus* teat**	85	32.7	32.7	2.6	26.8	39.1	31.1	34.4

Table 10 Descriptive statistics of the cycle threshold (Ct) values, including number (N), mean, median, standard deviation (Std Dev), minimum value (Min.), maximum value (Max.), 25th percentile (25th perc.) and 75th percentile (75th perc.). Ct value 40 is not included. In total, milk and teat skin samples were collected from 132 quarters.

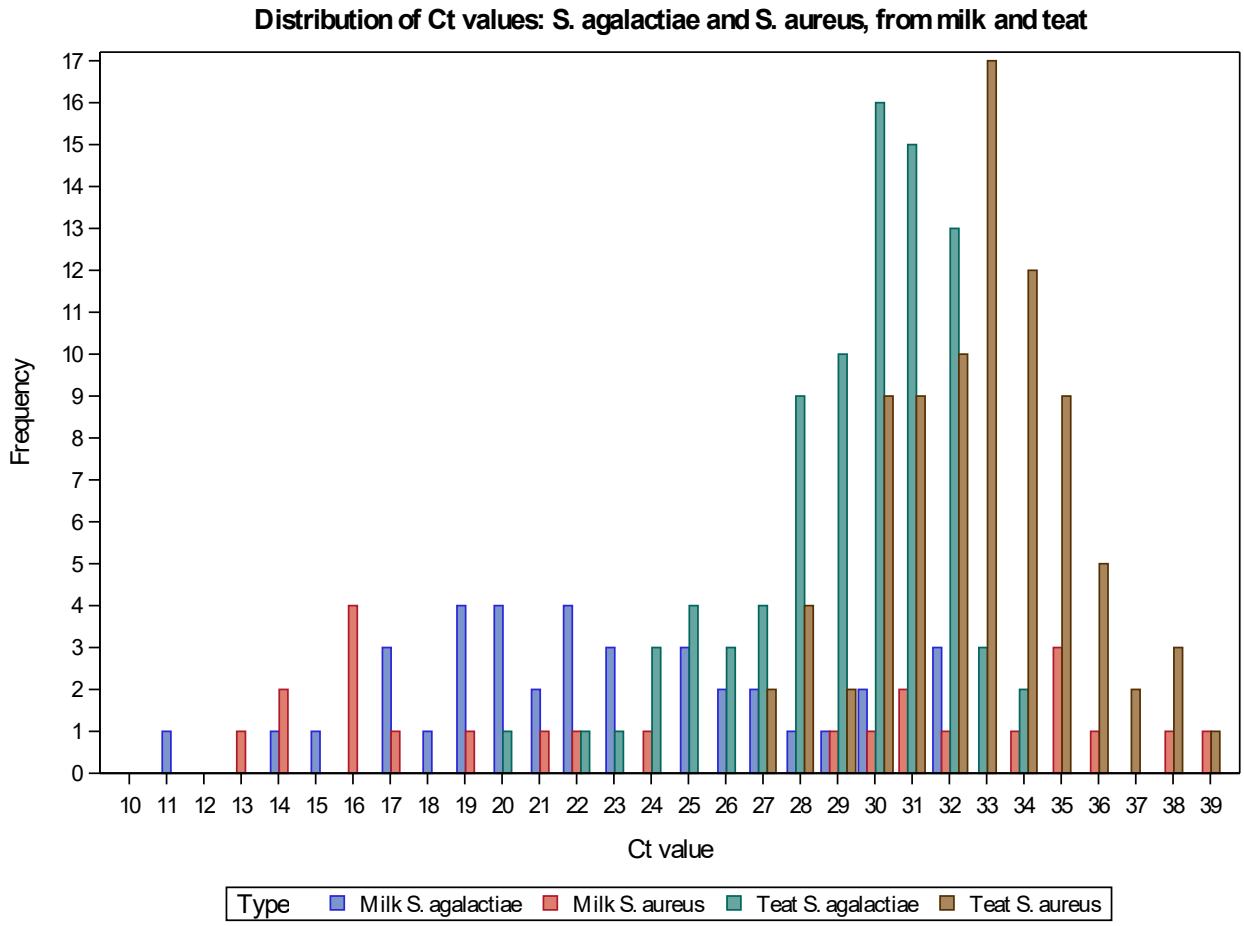


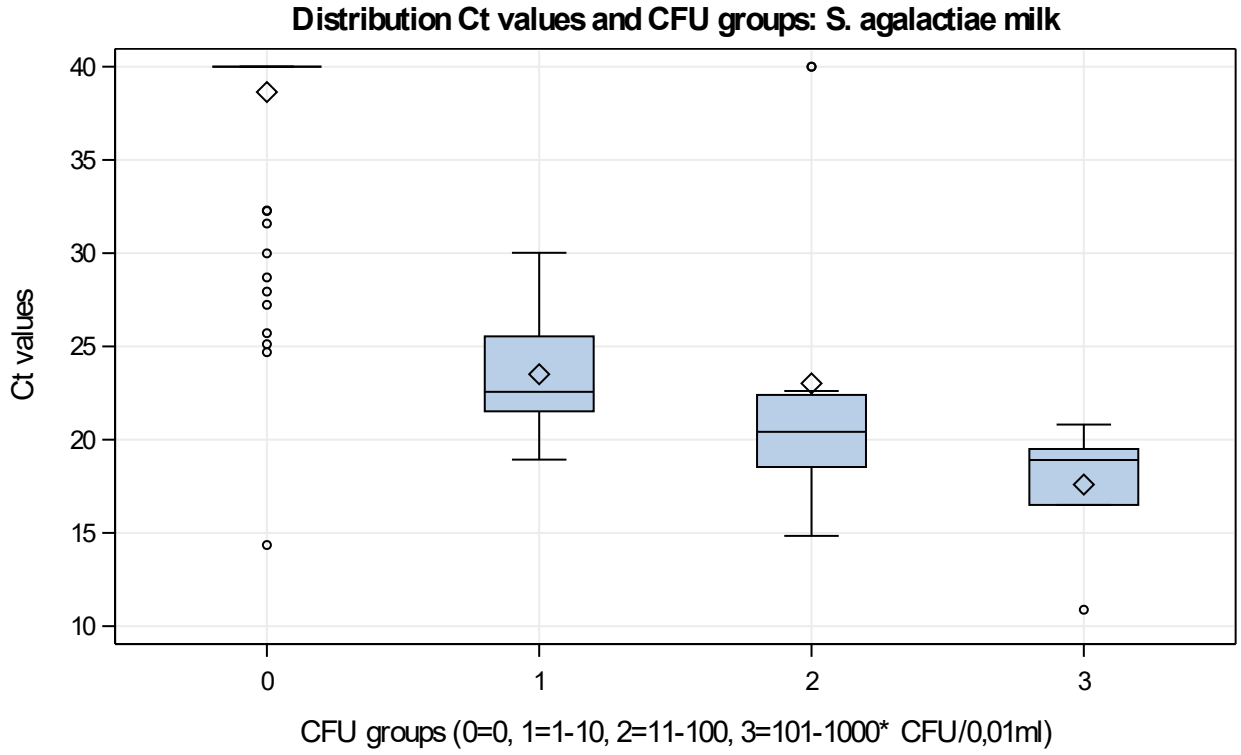
Figure 8 The distribution of cycle threshold (Ct) values in relation to pathogen (*S. aureus* or *S. agalactiae*) and sample type (milk or teat skin). Ct value 40 is not included. In total, milk and teat skin samples were collected from 132 quarters.

Distribution of Ct values in relation to CFU group

To investigate the association between Ct values and the number of CFU, the number of CFU was grouped by number in the same way as in part A. For milk samples, the number of CFU was stated per 0.01 ml milk whereas for teat skin samples, the number of CFU was stated per 0.1 ml of Ringer's solution.

S. agalactiae in milk samples

The distribution of Ct values in relation to CFU group for *S. agalactiae* in milk samples is seen in Figure 9. The figure strongly indicated an association between the Ct values and the CFU group. The Spearman Rank Correlation Coefficient of -0.81 [-0.86;-0.74] showed a strong and significant ($p < 0.0001$) negative relation between the two variables which implied that Ct values decreased with increasing CFU group. At Ct value cutoff ≤ 37, 11 samples (Table 11) were PCR positive but BC negative. The Ct values of BC negative samples ranged from 14 to 33 (Figure 9). Two samples with between 11 and 100 CFU were BC positive but PCR negative.

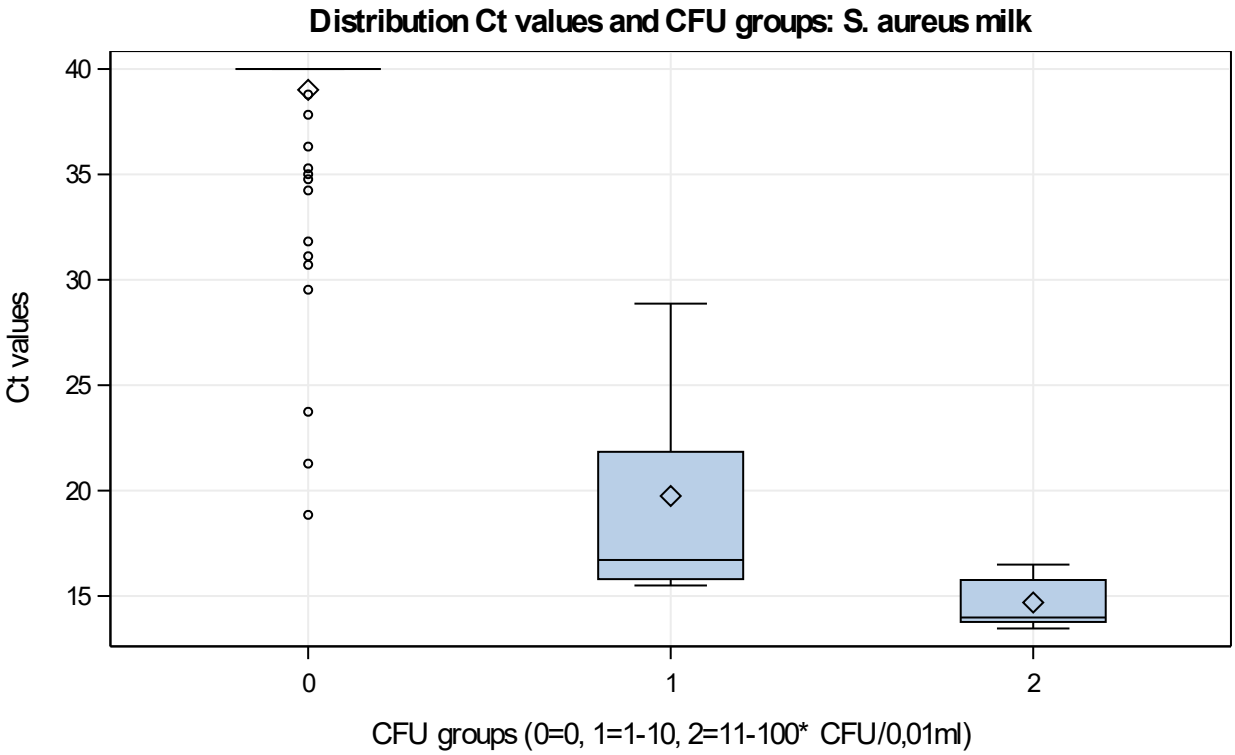


N	103	10	12	7
MIN	14.35	18.93	14.84	10.88
MAX	40	30.02	40	20.81
Median	40	22.565	20.42	18.91
Mean	38.639514563	23.509	23.016666667	17.592857143

Figure 9 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. agalactiae* in milk samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean. *CFU group 3 contained one observation with above 1000 CFU/0.01 ml (with a corresponding Ct value of 11).

S. aureus in milk samples

In Figure 10, the distribution of Ct values in relation to CFU group is shown for *S. aureus* in milk samples. This figure indicated an association between the Ct values and the CFU group. The Spearman Rank Correlation Coefficient of -0.68 [-0.76;-0.57] showed a strong and significant ($p < 0.0001$) negative relation between the two variables implying that Ct values decreased with increasing CFU group. At Ct value cutoff ≤ 37, 12 samples were PCR positive but BC negative (Table 11).



	0	1	2
N	122	5	5
MIN	18.85	15.5	13.46
MAX	40	28.87	16.49
Median	40	16.71	13.98
Mean	39.010655738	19.744	14.692

Figure 10 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. aureus* in milk samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean. *CFU group 2 contained one observation with above 200 CFU/0.01 ml (with a corresponding Ct value of 16).

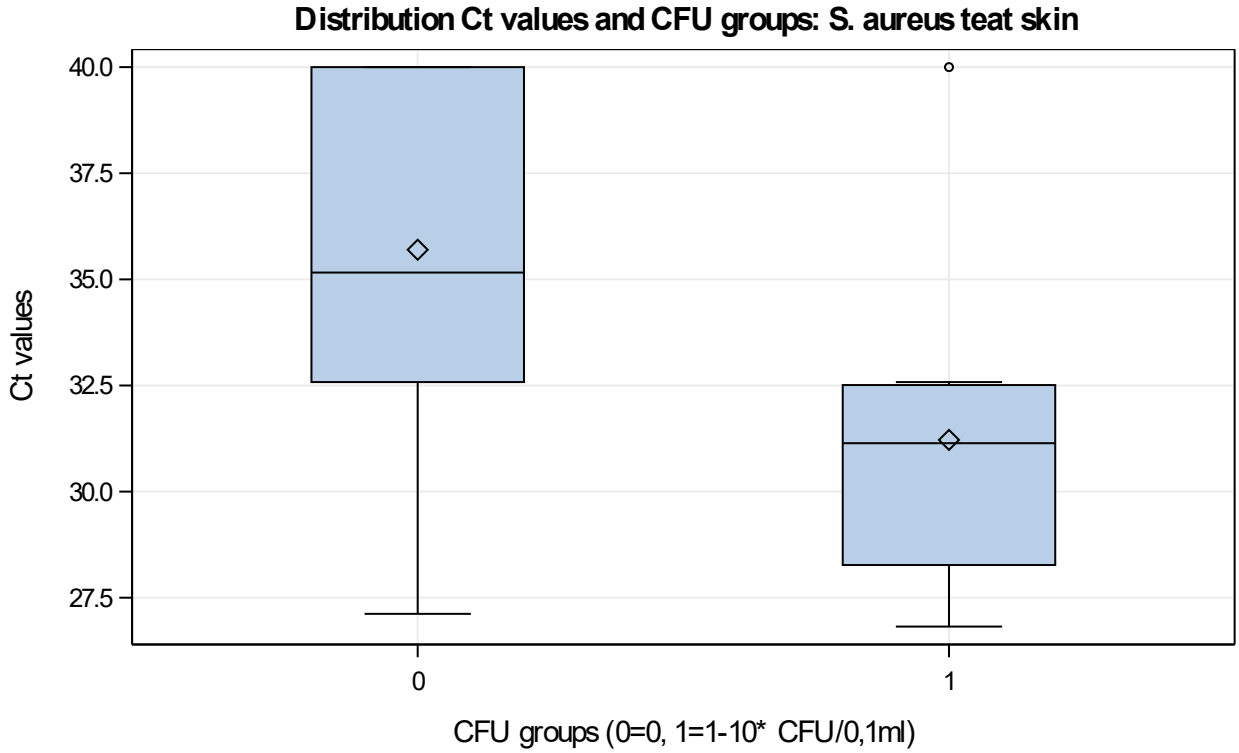
S. agalactiae in teat skin samples

Whereas 85 teat skin samples had a positive PCR test for *S. agalactiae* (Ct value cutoff ≤ 37), only one quarter was positive with BC (Table 11) which is why no analysis based on the graphical illustration of the association between Ct values and CFU was made. Hence 84 samples were positive with PCR but negative with BC. The distribution of Ct values in BC negative samples is seen in Figure B in Appendix IV.

S. aureus in teat skin samples

In Figure 11, the distribution of Ct values in relation to CFU group is shown for *S. aureus* in teat skin samples. Since there was only one CFU group (CFU group 1) besides CFU group 0, the foundation for concluding an association between Ct values and CFU group was insufficient. The Spearman Rank Correlation Coefficient of -0.31 [-0.46;-0.15] implied a weak, yet significant (p = 0.0003), negative relation between the Ct values and the CFU group. Seventy samples were

positive with PCR but negative with BC (Table 11 at Ct value cutoff ≤ 37), but the Ct values were high. One sample was positive with BC (between one and 10 CFU) but negative with PCR.



	0	1
N	121	11
MIN	27.12	26.82
MAX	40	40
Median	35.16	31.14
Mean	35.697768595	31.217272727

Figure 11 The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. aureus* in teat skin samples. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean. *CFU group 1 contained one observation with 51 CFU/0.1 ml (with a corresponding Ct value of 33).

Diagnostic test properties of PCR and BC

In the following, results from the McNemar test, kappa, LCA, and comparison of PCR relative to BC (regarding teat skin samples) are presented. The results are presented for both part A and B.

McNemar test and kappa

Comparison of the two methods (PCR and BC) for detection of either *S. aureus* or *S. agalactiae* on either teat skin or in milk was performed using both McNemar test and kappa. The McNemar test evaluates the difference in the probability for being positive using two methods whereas kappa is a measure of agreement adjusted by the agreement by chance (Ersbøll et al. 2004). Both the χ^2 values and p-values of the McNemar test and the kappa coefficients with 95 % CI for three different Ct value cutoffs are shown in Table 12. Underlying data are shown in Table 11.

			Ct < 40 and CFU ≥ 1			Ct ≤ 37 and CFU ≥ 1			Ct ≤ 32 and CFU ≥ 1		
			PCR 1	PCR 0	Total	PCR 1	PCR 0	Total	PCR 1	PCR 0	Total
Part A	Teat *S. agalactiae*	BC 1	1	0	1	1	0	1	1	0	1
		BC 0	51	235	286	51	235	286	40	246	286
		Total	52	235	287	52	235	287	41	246	287
	Milk *S. agalactiae*	BC 1	19	0	19	19	0	19	19	0	19
		BC 0	21	247	268	21	247	268	18	250	268
		Total	40	247	287	40	247	287	37	250	287
	Teat *S. aureus*	BC 1	7	16	23	7	16	23	1	22	23
		BC 0	38	226	264	35	229	264	14	250	264
		Total	45	242	287	42	245	287	15	272	287
	Milk *S. aureus*	BC 1	19	3	22	19	3	22	18	4	22
		BC 0	10	255	265	10	255	265	6	259	265
		Total	29	258	287	29	258	287	24	263	287
Part B	Teat *S. agalactiae*	BC 1	1	0	1	1	0	1	1	0	1
		BC 0	84	47	131	84	47	131	76	55	131
		Total	85	47	132	85	47	132	77	55	132
	Milk *S. agalactiae*	BC 1	27	2	29	27	2	29	27	2	29
		BC 0	11	92	103	11	92	103	9	94	103
		Total	38	94	132	38	94	132	36	96	132
	Teat *S. aureus*	BC 1	10	1	11	10	1	11	7	4	11
		BC 0	75	46	121	70	51	121	24	97	121
		Total	85	47	132	80	52	132	31	101	132
	Milk *S. aureus*	BC 1	10	0	10	10	0	10	10	0	10
		BC 0	14	108	122	12	110	122	7	115	122
		Total	24	108	132	22	110	132	17	115	132

Table 11 Findings from polymerase chain reaction (PCR) and bacteriological culture (BC) with different cycle threshold (Ct) value cutoffs (< 40, ≤ 37, ≤ 32) and colony forming units (CFU) ≥ 1. 1: Positive with the given method, 0: Negative with the given method.

Part	Pathogen	Sample	Ct < 40 and CFU ≥ 1		Ct ≤ 37 and CFU ≥ 1		Ct ≤ 32 and CFU ≥ 1	
			McNemar χ^2 (P)	**Kappa coeff. [95 % CI]**	**McNemar χ^2 (P)**	**Kappa coeff. [95 % CI]**	**McNemar χ^2 (P)**	**Kappa coeff. [95 % CI]**
A	*S. agalactiae*	Teat	49.02 (0.000)	0.031 [0-0.091]	49.02 (0.000)	0.031 [0-0.091]	38.03 (0.000)	0.041 [0-0.119]
		Milk	19.05 (0.000)	0.61 [0.461-0.757]	19.05 (0.000)	0.61 [0.461-0.757]	16.06 (0.000)	0.65 [0.500-0.795]
	S. aureus	Teat	8.17 (0.004)	0.11 [0-0.247]	6.35 (0.012)	0.13 [0-0.265]	1.36 (0.243)	-0.011 [-0.124-0.101]
		Milk	2.77 (0.096)	0.72 [0.577-0.864]	2.77 (0.096)	0.72 [0.577-0.864]	0.10 (0.752)	0.76 [0.623-0.905]
B	*S. agalactiae*	Teat	82.01 (0.000)	0.0084 [0-0.025]	82.01 (0.000)	0.0084 [0-0.025]	74.01 (0.000)	0.001 [0-0.032]
		Milk	4.92 (0.027)	0.74 [0.611-0.872]	4.92 (0.027)	0.74 [0.611-0.872]	3.27 (0.070)	0.78 [0.652-0.901]
	S. aureus	Teat	70.12 (0.000)	0.071 [0.009-0.133]	65.13 (0.000)	0.086 [0.017-0.155]	12.89 (0.000)	0.24 [0.055-0.425]
		Milk	12.07 (0.001)	0.54 [0.336-0.742]	10.08 (0.001)	0.58 [0.376-0.787]	5.14 (0.023)	0.71 [0.516-0.911]

Table 12 Comparison of polymerase chain reaction (PCR) and bacteriological culture (BC) for three different cycle threshold (Ct) value cutoffs (< 40, ≤ 37, ≤ 32) and colony forming units (CFU) ≥ 1 using McNemar test and kappa. Confidence intervals (CI) for kappa coefficients (coeff.) are shown in brackets. Moderate agreement: 0.4 < kappa ≤ 0.6, good agreement: 0.6 < kappa ≤ 0.8 (Landis & Koch 1977). McNemar statistic values (χ^2) and corresponding p-values (P) are shown, the probability of being tested positive is significantly different for the two methods if P ≤ 0.05.

The agreement (kappa) between the two methods (PCR and BC) was similar for Ct value cutoff < 40 and Ct value cutoff ≤ 37 regarding *S. agalactiae* in both milk and teat skin samples and in both part A and B. The agreement between the two methods was similar for Ct value cutoff < 40 and Ct value cutoff ≤ 37 regarding *S. aureus* in milk samples in part A.

For all Ct value cutoffs (< 40, ≤ 37, ≤ 32) for *S. aureus* in milk samples in part A, there was no significant difference (P > 0.05) in the probability of being tested positive using the two methods.

Moderate (0.4 < kappa ≤ 0.6) and good (0.6 < kappa ≤ 0.8) agreement (Landis & Koch 1977) between the two methods are seen only for milk but in both part A and B and for both *S. aureus* and *S. agalactiae*. Kappa was higher for *S. agalactiae* in milk samples from part B compared to part A while kappa was higher for *S. aureus* in milk samples from part A compared to part B.

For milk samples, there might have been an association between the Ct value cutoffs, the McNemar χ^2 values, p-values and kappa: Decreasing Ct value cutoffs (from ≤ 37 to ≤ 32) resulted in lower χ^2 values and corresponding higher p-values (except *S. agalactiae* in milk samples in part A) for McNemar and an increased kappa. For teat skin samples, there was no indication of a similar association since the results were inconclusive (decreasing Ct value cutoffs resulted in both increased and decreased kappa coefficients for both pathogens, and p-values for McNemar only decreased for S. aureus in part A). On the other hand, the McNemar χ^2 values all decreased with decreasing Ct value cutoff (from ≤ 37 to ≤ 32).

Latent class analysis

A total of 287 quarters in part A and 132 quarters in part B from eight herds were included in the LCA. Two populations were created based on AMS type (Lely or DeLaval), and the test results from PCR and BC in part A and B are shown in Table 13. Population 1 consisted of herds with Lely robots, and population 2 consisted of herds with DeLaval robots (Table 2).

Sample type	Visit type	Pathogen	Population	Test combinations				Total
				PCR + BC +	PCR + BC -	PCR - BC +	PCR - BC -	
Milk	Part A	*S. agalactiae*	Population 1 (Lely)	10	20	0	184	214
			Population 2 (DeLaval)	9	1	0	63	73
		S. aureus	Population 1 (Lely)	7	5	1	201	214
			Population 2 (DeLaval)	12	5	2	54	73
	Part B	*S. agalactiae*	Population 1 (Lely)	12	9	2	74	97
			Population 2 (DeLaval)	15	2	0	18	35
		S. aureus	Population 1 (Lely)	2	6	0	89	97
			Population 2 (DeLaval)	8	6	0	21	35

Table 13 Test results of polymerase chain reaction (PCR) and bacteriological culture (BC) for diagnosis of intramammary infections (IMI) with *S. agalactiae* and *S. aureus* in two populations based on automatic milking system (AMS) type (Lely or DeLaval) at cycle threshold (Ct) value cutoff ≤ 37 and colony forming units (CFU) ≥ 1 at two rounds of herd visits (part A and B).

Even though the total number of observations in the two populations differed for both part A and B, the number of samples positive with the two methods (PCR and BC) and for the two different pathogens was reasonably similar (data not shown). The test estimates based on the median (%) are shown in Table 14. Underlying data is shown in Appendix VI.

Sample type	Visit type	Pathogen	Test estimates (median in %)				Prevalence (median in %)	
			Se_{PCR}	Se_{BC}	Sp_{PCR}	Sp_{BC}	P1 (Lely)	P2 (DeLaval)
Milk	Part A	*S. agalactiae*	96.4 [82.0-99.9]	82.4 [43.6-99.3]	93.4 [89.2-99.2]	99.7 [98.5-99.9]	6.7 [3.1-15.0]	13.6 [7.0-22.9]
		S. aureus	87.6 [68.2-98.8]	74.0 [51.8-95.6]	98.2 [95.1-99.9]	99.4 [97.5-99.9]	4.9 [2.2-9.1]	25.1 [15-37.2]
	Part B	*S. agalactiae*	95.4 [81.7-99.8]	88.6 [65.7-99.5]	91.4 [83.6-98.6]	97.9 [93.2-99.9]	15.6 [8.3-26.1]	46.1 [29.9-63.1]
		S. aureus	93.2 [68.3-99.7]	57.7 [31.1-88.7]	94.7 [87.8-99.5]	99.4 [96.6-99.9]	4.5 [0.9-12.2]	38.4 [20.9-58.1]

Table 14 Estimates and 95 % posterior credibility intervals (PCI) (in brackets) of prevalence, sensitivity (Se_{PCR}) and specificity (Sp_{PCR}) for Mastit4 qPCR Assay and sensitivity (Se_{BC}) and specificity (Sp_{BC}) for bacteriological culture (BC) for diagnosis of intramammary infections (IMI) with *S. agalactiae* and *S. aureus*, determined with latent class analysis (LCA) at cycle threshold (Ct) value cutoff ≤ 37 and colony forming units (CFU) ≥ 1 in two populations (P) (Lely and DeLaval) at two rounds of herd visits (part A and B). In part B, PCR swabs was carried out directly on farm.

For milk samples, sensitivity of PCR (Se_{PCR}) and sensitivity of BC (Se_{BC}) were lower for *S. aureus* than for *S. agalactiae* in both part A and B. The Se_{BC} was higher in part B than in part A for *S. agalactiae* whereas the Se_{BC} was lower in part B than in part A for *S. aureus*. The Se_{PCR} for *S. agalactiae* was similar in part A and B whereas the Se_{PCR} for *S. aureus* was slightly higher in part

B compared to part A. In general, Se_{PCR} was higher than Se_{BC}. The PCI for Se_{BC} were generally wider than for Se_{PCR}. The specificity of BC (Sp_{BC}) was generally higher than the specificity of PCR (Sp_{PCR}), but both were high and with narrow PCI.

The estimated prevalence represented the prevalence of RH quarters (part A) and quarters (part B) with *S. agalactiae* or *S. aureus* IMI, respectively, among cows with high SCC in herds with known positive status for *S. agalactiae* and with Lely or DeLaval robots, respectively. The prevalence in the two populations differed markedly between part A and B (except the prevalence of *S. aureus* in Lely herds), ranging from 6.7 to 15.6 % for *S. agalactiae* in Lely herds (population 1) and from 13.6 to 46.1 % for *S. agalactiae* and from 25.1 to 38.4 % for *S. aureus* in DeLaval herds (population 2). Estimation of prevalence in part B was necessary to be able to carry out LCA but cannot be interpreted meaningfully because of the selection criteria. Due to the selection criteria, where only positive quarters were targeted, a higher prevalence in part B was expected.

Sensitivity and specificity using bacteriological culture as a reference standard

Due to the low number of teat skin samples positive for *S. agalactiae* and *S. aureus*, LCA was not applicable after dividing the data into two populations. Therefore, sensitivity, specificity, PPV, and NPV for Mastit4 qPCR Assay as a diagnostic test for detection of teat skin colonization with *S. aureus* or *S. agalactiae* were calculated using BC as a reference standard. Results are shown in Table 15.

Visit type	Sample type	Pathogen	Population prevalence P (%)	Se_{PCR} (%)	Sp_{PCR} (%)	PPV (%)	NPV (%)
A	Teat skin	*S. agalactiae*	0.3 [0.01-2.2]	100 [5.5-100]	82.2 [77.1-86.3]	1.9 [0.1-11.6]	100 [98.0-100]
		S. aureus	8.0 [5.3-11.9]	30.4 [14.1-53.0]	86.7 [81.9-90.5]	16.7 [7.5-32.0]	93.5 [89.4-96.1]
B	Teat skin	*S. agalactiae*	0.8 [0.03-4.8]	100 [5.5-100]	35.9 [27.8-44.8]	1.2 [0.06-7.3]	100 [90.6-100]
		S. aureus	8.3 [4.4-14.8]	90.9 [57.1-99.5]	42.1 [33.3-51.5]	12.5 [6.5-22.2]	98.1 [88.4-99.9]

Table 15 Sensitivity (Se_{PCR}) and specificity (Sp_{PCR}), positive predictive value (PPV), negative predictive value (NPV), and population prevalence (P) calculated for Mastit4 qPCR Assay as a test for detection of teat skin colonization with *S. aureus* or *S. agalactiae* using bacteriological culture (BC) as a reference standard at two rounds of herd visits (part A and B). Confidence intervals (CI) are shown in brackets. Cycle threshold (Ct) value cutoff ≤ 37 and colony forming units (CFU) ≥ 1.

The calculated population prevalence represented the prevalence of RH quarters (part A) and quarters (part B) with teat skin colonization with *S. aureus* or *S. agalactiae* among cows with high SCC in herds with known positive status for *S. agalactiae*. It is important to comment that the

population prevalence in part B was overestimated due to the selection criteria. Se_{PCR} was higher for *S. agalactiae* compared to *S. aureus* in both part A and B, but the CI for Se_{PCR} for *S. agalactiae* were very wide, indicating unreliable results. Sp_{PCR} was lower for *S. agalactiae* compared to *S. aureus* in both part A and B. Se_{PCR} for *S. agalactiae* was similar comparing part A with part B whereas Se_{PCR} for *S. aureus* was markedly higher in part B compared to part A. The PPV for all scenarios was generally low whereas the NPV was generally high. The few positive observations with BC for both *S. agalactiae* and *S. aureus* on teat skin were reflected in the PPV and NPV: Few positive cases will make it hard to detect true positive cases, and many negative cases will make it easy to detect true negative cases.

Discussion

To our knowledge, this is the first study comparing the findings with BC and PCR regarding teat skin colonization. The finding of few *S. agalactiae* BC positive teat skin samples compared to several PCR positive teat skin samples is a unique finding and initiates an important discussion about underlying 'disease' definitions, and what PCR and BC really detect.

Other unique findings of this study involve the use of LCA to estimate the diagnostic test properties of BC and PCR for diagnosis of IMI with *S. aureus* and *S. agalactiae* in herds with AMS, the fact that data was collected at two rounds of herd visits, and that a PCR swab was used for both milk and teat skin samples. The finding of quarters with positive teat skin samples with either *S. aureus* or *S. agalactiae* in both part A and B could indicate that teat skin colonization rather than contamination occurs in some quarters, but further studies on teat skin are needed.

Using LCA, the sensitivity and specificity of BC and PCR for the diagnosis of IMI with either *S. aureus* or *S. agalactiae* were estimated in the absence of a perfect reference test. Due to few or zero positive samples from teat skin, sensitivity and specificity of BC and PCR for the detection of *S. aureus* or *S. agalactiae* on teat skin were estimated using BC as a reference standard. Kappa was calculated to analyze the agreement between PCR and BC whereas the McNemar test was used to evaluate the difference in the probability of being tested positive with the two methods. The association between Ct values and CFU groups was analyzed using box-and-whisker plots and Spearman Rank Correlation Coefficients.

In the following, we will first discuss our study design and the statistical analyses before our results from milk and teat skin samples, respectively, are discussed in detail. Finally, the differences in the findings of PCR and BC are discussed from a biological perspective.

Study design and statistical analyses

Study design

Selection criteria

Herds: Only eight herds were willing to participate which could lead to potential selection bias. Willingness to participate could be influenced by the prevalence in the herd thus herds with either high or low prevalence were more willing to participate. Since we found the proportion of positive results to vary greatly from herd to herd, this specific selection bias was not suspected. Even

though herds were selected based on a positive status for *S. agalactiae*, we still identified a satisfactory amount of positive *S. aureus* samples from both milk and teat skin and with both BC and PCR as seen in Table 3, 4, 6 and 7.

Cows: To increase the probability of detecting cows with subclinical *S. aureus* or *S. agalactiae* IMI, the cows were selected based on a high (> 200.000 cells/ml) SCC. Erskine et al. (1988) found that the prevalence of IMI with *S. agalactiae* and *S. aureus* was significantly higher in herds with high (> 700.000 cells/ml) SCC compared to herds with low (≤ 150.000 cells/ml) SCC. Not all *S. agalactiae* BC positive cows have a composite SCC > 200.000 cells/ml which is why SCC as the sole indicator of infection status is unreliable (Mahmmod et al. 2015). Despite these findings, we regard our cow selection criteria as the most reasonable and rational compared to selection not based on (or based on a low) SCC.

Teat: In our study, only RH quarters were investigated (part A). Findings regarding which quarter position (front or hind) and quarter side (left or right) are the most frequently infected are inconsistent. Hammer et al. (2012) found no difference neither between right and left quarters nor between front and hind quarters, unlike many other studies suggesting that hind quarters are more frequently infected than front quarters (Miltenburg et al. 1996; Barkema et al. 1998; Shpigel et al. 1998). Tolosa et al. (2015) found that right quarters are more frequently infected than left quarters ($p = 0.01$). This consideration of more studies indicates that if any quarter, the RH quarter is more frequently infected and therefore chosen for our study. Naturally, if all four quarters were included in the study, more positive samples would have been expected.

Independency between interpretation of PCR and BC

The people responsible for the PCR analysis differed from the people responsible for BC, and often the results from PCR were received after the reading of plates, thereby eliminating potential bias regarding interpretation of results. However, sometimes, the results from PCR were received before we discarded the plates, and in three cases, we detected *S. agalactiae* on the plates afterwards, only because the received results from PCR were positive and we double-checked the plates. Hence, the Se_{BC} was falsely increased. In part B, the quarters were selected based on positive results from part A. Hence, we expected some quarters to be positive, and the plates were perhaps more thoroughly read which could also lead to a falsely increased Se_{BC}. Therefore, findings from especially part B were less comparable to other studies and should be interpreted with caution.

Standardization

Even though a training session was carried out before the sampling period, it was difficult to standardize sampling of the teat skin regarding pressure and sampling area. Although interobserver and intraobserver variability cannot be avoided, the same people collected the teat skin samples throughout the study, thereby reducing the variability. It is hard to imagine that the study could have been completed more standardized under field conditions because of the different farm environments and the more or less kicking cows.

Sampling period

Unfortunately, several cows positive in part A were negative in part B (Table 8 and 9). When the results from part A were known, the farmers received the results. Even though the farmers were asked not to, treatment with antibiotics could explain the proportion of negative cows in part B. Time from part A to part B varied between herds, from seven to 22 days, why another explanation of the proportion of negative cows could be self-cure or intermittent shedding as described for both *S. agalactiae* and *S. aureus* (Anderson 2012; Svennesen et al. n.d.). Shortening the time between part A and part B was not always practicably because firstly, we needed the results from both PCR and BC before planning the revisit and secondly, the day for the revisit should fit into the farmer's calendar.

Sampling procedures

Immersion of PCR swab

In part B, the PCR swab for milk sample analysis was immersed into the milk sample immediately after sampling, i.e. before plating the next day. If milk samples are not vortexed before the PCR swab is immersed, a layer of fat will form in the top of the tube wherein staphylococci will accumulate (Katholm 2017, personal communication). Because the PCR swab was only immersed into the top of the tube, this could have caused a higher Se_{PCR} for *S. aureus* in milk samples in part B.

Dimensions of PCR swab

The disagreement between the number of positive teat skin samples between PCR and BC in part B could be explained by the difference in the dimensions of the used swabs. The length of the PCR swab was two cm, whereas the rayon wet-dry swabs for BC were only one cm (two swabs were used though). According to unpublished data regarding genetic evaluation of Danish cattle, the average teat diameter (middle part) for Holstein cattle is 2.3 cm, hence the area from where the PCR swab was sampled was 14.4 cm^2, and the area from where the wet-dry swabs for BC were

sampled was 7.2 cm^2 (two swabs used though). The sum of the areas was the same, but the PCR swab covered one larger area, whereas the wet-dry swabs covered the same smaller area twice why a smaller number of bacteria sampled by the wet-dry swabs was expected.

Laboratory procedures

Plates

Usefulness of modified Edward's medium

According to results from BC in our study, the prevalence of *S. agalactiae* on teat skin from RH quarters among cows with high SCC in herds with positive *S. agalactiae* disease status was 0.3 % (Table B in Appendix VII). To our knowledge, the presence of *S. agalactiae* on teat skin has not been investigated and hence no basis for comparison of prevalence exists. The reasons for low prevalence could either be that *S. agalactiae*, in contrast to *S. aureus*, does not colonize the teat skin (will be discussed later), or a low detection rate because of the chosen sampling and laboratory procedures along with the experience of the investigators. The usefulness of the modified Edward's medium has in our opinion been questionable due to its variable transparency, making evaluation of CAMP reaction difficult.

Inoculum amount

To determine if the inoculum used for BC was insufficient in detecting *S. agalactiae*, we increased the amount of inoculum so that both 100 and 200 µl from teat skin samples were streaked on modified Edward's medium, and both 10 and 100 µl from milk samples were streaked on modified Edward's medium. This was done for herd 1 and 2 in part B. The number of positive samples were not increased (data not shown), showing that the initially chosen amount of inoculum was appropriate for the type of samples, and for this reason we continued the laboratorial analyses with 100 and 10 µl from teat skin and milk samples, respectively.

Dilution series

According to results from BC in our study, the prevalence of *S. aureus* on teat skin from RH quarters among cows with high SCC was 8 % (Table B in Appendix VII). With the same wet-dry method, Paduch & Krömker (2011) found *S. aureus* on 61.4 % of teat skin samples from clinically healthy cows. They included a dilution series which is recommendable, especially when agar plates are overgrown (Krömker 2017, personal communication). If this study should be redone, inclusion of a dilution series is a possibility (since we had several overgrown plates) but because

the estimated number of CFU was already low, the risk of worsening the detection rate by further dilution must be considered.

Choice of plates

Neither the calf blood agar nor the selective medium (SA*Select*™ medium and modified Edward's medium for *S. aureus* and *S. agalactiae*, respectively) worked perfectly as a growth medium since the findings of the plates were inconsistent, i.e. sometimes the modified Edward's medium or the SA*Select*™ medium were positive and the blood agar negative, and sometimes the opposite was the case (Appendix III). Therefore, no growth medium was superior to the other. In part B, only calf blood agar was used for the detection of *S. aureus* since the SA*Select*™ medium was mainly used for identification of CNS in part A (as part of Yasser Mahmmod's postdoc). In hindsight, both calf blood agar and SA*Select*™ medium should have been included to increase the sensitivity of BC for the detection of *S. aureus* in samples from part B. Partly because of the disagreement between the different growth media but also because the calf blood agar was often overgrown when positive with *S. aureus*, making the isolation and identification difficult.

Quality check of laboratory procedures

Our laboratory procedures, e.g. the quality of our plates, the choice of plates, and the inoculum amount were all sources of concern, especially for teat skin samples, since so few positive samples were found with BC. For milk samples, however, laboratory procedures were never a source of concern, since, among other things, overgrowth was no issue and findings were comparable to those of other studies (Cederlöf et al. 2012; Mahmmod et al. 2013a; Mahmmod et al. 2013b). In contrast to analyses of milk samples, no common guidelines for teat skin samples are available, explaining why the optimal choice of plates does not yet exist. By testing different inoculum amounts and using selective media as well as calf blood agar, optimal laboratory procedures have been investigated. The presence of CNS on the teat skin contributes to another plausible explanation for the few BC positive findings. Even with these considerations, we have often seen an association between the cleanliness of the herd and the growth on the agar plates, indicating that low prevalence equals good mastitis management which could further indicate that our sampling and laboratory procedures were acceptable after all. Still, laboratory procedures for teat skin samples need to be investigated further.

Confirmation

All suspected *S. aureus* and *S. agalactiae* colonies from BC were confirmed by MALDI-TOF MS and either MALDI-TOF MS or latex agglutination test, respectively. Identification of bacteria

based only on morphological characteristics requires laboratory experience why misinterpretation can easily occur and lead to false positive or false negative findings. For instance, the colony morphology of *S. aureus* from especially teat skin on calf blood agar varied between herds and even cows and quarters, hence *S. aureus* could easily be missed by an inexperienced person, thereby reducing the sensitivity of BC. Therefore, the intraobserver and interobserver variability was lowered by confirmation with MALDI-TOF MS or latex agglutination test of all suspected colonies.

Sometimes, suspected *S. aureus* colonies from teat skin samples were completely overgrown by other bacteria or fungi, especially on calf blood agar. In these cases, isolation and identification by MALDI-TOF MS was difficult or even impossible. In continuation of this, confirmation with MALDI-TOF MS had some limitations as well. Only one suspected colony from each plate was submitted for MALDI-TOF MS, hence the importance of a pure culture to prevent identification of the wrong bacterium. Furthermore, the outcome of the analysis depended on the operator of the machine. If the wrong colony was picked or too little colony material was placed on the sample slide, false negative results appeared. A final limitation was the database of the MALDI-TOF MS; only bacteria available in the database could be identified.

The agglutination test kit used for confirmation had a sensitivity and a specificity of 89.1 and 97.8 %, respectively (PathoDxtra Strep Grouping Kit 2013). An insufficient number of colonies for extraction may result in false negative results (PathoDxtra Strep Grouping Kit 2013), hence a pure culture is important. In our study, all subcultures submitted for agglutination test were pure and with sufficient growth. If a negative result appeared from a suspected positive subculture, the test was repeated on the same subculture or on a new subculture the day after.

PCR is on the other hand based on a more objective bacterial identification since procedures are easier to standardize. Removal of supernatants are included several times during the DNA extraction step which is one of the few steps that can be hard to standardize. At DNA Diagnostic A/S, this was performed by a vacuum system, assuring standardization. The employees at DNA Diagnostic A/S were skilled, since they daily work with PCR analysis.

Lack of confirmation in part A

Thirty-two milk and 20 teat skin samples, primarily from herd 1 to 3, were assumed *S. aureus* positive by visual confirmation (colony form, color, presence of hemolysis) but never confirmed by MALDI-TOF MS. On May 1st, 2017, the 52 samples were thawed and restreaked following the same laboratory procedures as the first streaking. Since only *S. aureus* was suspected, all

samples were streaked on both SA*Select*™ medium and calf blood agar. *S. aureus* was only detected in 16 of the milk and none of the teat skin samples. We do not know if the remaining 36 samples were really *S. aureus* positive at first and then bacteria were killed during freezing, thereby reducing the sensitivity of BC, or if they were never positive. Freezing of milk samples has shown an increase in the sensitivity of detecting *S. aureus* (Villanueva et al. 1991; Godden et al. 2002). If this is also the case for Ringer's solution, the samples were probably never positive.

Statistical analyses

Model assumptions: Latent class analysis

According to the Hui & Walter paradigm (1980), it is important that test characteristics (sensitivity and specificity) of PCR and BC are constant across populations. The splitting into two suitable populations based on AMS type raises the question whether the AMS type could influence the test characteristics. Due to logistic reasons, sampling of the two DeLaval herds took place at last which could have increased the sensitivity of especially BC since we had more sampling and laboratory experience at that point.

Another important assumption for the LCA is that the prevalence in the two populations should differ. According to Bennedsgaard & Katholm (2014), herds with Lely robots had a significantly higher prevalence of *S. agalactiae* compared to herds with DeLaval robots based on BTM samples from Danish herds (year 2009 – 2012). This finding supports our decision to split the population based on AMS type, even though estimation of prevalence within herds was not a part of the study of Bennedsgaard & Katholm (2014).

In the attempt to make the most suitable population split, we tried sorting after geographical location (south, north), herd size (0 – 300 cow-years, > 300 cow-years) and finally AMS type. In all versions, limitations of the model existed. For population split based on herd size and geographical location, test estimates of population prevalence were too similar, thus conflicting with the Hui & Walter paradigm (1980). For population split based on AMS type, test characteristics in the two populations were suspected to be varying due to the previously mentioned difference in sampling experience. Whether this suspicion is entitled is hard to judge since the higher prevalence found in population 2 (DeLaval herds) (Table 14) could be due to both more sampling experience or simply a higher occurrence of *S. agalactiae* and *S. aureus* mastitis in population 2. If a difference in sensitivity between the two populations was the case, a bias towards the population estimate supported by the most data would have occurred (Toft et al. 2005).

In our data, this would have been population 2 (DeLaval herds) with prevalence ranging from 13.6 to 46.1 %.

Another important aspect to consider is the fact that two different pathogens, *S. agalactiae* and *S. aureus*, with different disease prevalence as previously mentioned, are included in this LCA. Population split based on two pathogens acting differently from herd to herd is complicated which can explain why no optimal split was found. Of the different population splits, sorting by AMS type best fulfilled the Hui & Walter assumptions of the model.

Milk samples

Association between Ct values and CFU group

As expected, we found an association between the Ct values and the CFU groups, with a higher correlation in milk samples compared to teat skin samples. For milk samples, the correlations differed slightly between part A and B and ranged between -0.7 to -0.81 and -0.68 to -0.75 for *S. agalactiae* and *S. aureus*, respectively, and indicated that the greater the number of CFU in the sample, expressed as an increase in CFU group, the lower the Ct value. This was in agreement with Bennedsgaard et al. (2016) who reported an exponential relation between Ct values and CFU in milk samples.

Diagnostic test properties of PCR and BC

Steele et al. (2017) showed a high agreement (kappa = 0.96) between PCR and BC for the detection of *S. aureus* in milk samples, indicating that detection with PCR was comparable to BC. At Ct value cutoff ≤ 37, our results showed a moderate (kappa = 0.58) to good (kappa = 0.72) agreement between PCR and BC for the detection of *S. aureus* in milk samples in part B and A, respectively. In addition, according to the McNemar test, we found no difference between PCR and BC in the probability of being tested positive for *S. aureus* in part A. The higher agreement showed by Steele et al. (2017) compared to our findings could be explained by the greater amount of milk used for DNA extraction. In general, according to our study, the agreement between PCR and BC for the detection of either *S. aureus* or *S. agalactiae* in milk samples was good. When comparing the kappa coefficients between part A and B, kappa was higher for *S. agalactiae* in milk samples from part B compared to part A, while kappa was higher for *S. aureus* in milk samples from part A compared to part B. These findings could be biased because of our selection criteria: We made quarters positive with *S. agalactiae* a priority over quarters positive with *S. aureus*, and thus agar plates were thoroughly read in search for *S. agalactiae* positive quarters.

Using LCA at Ct value cutoff ≤ 37, in part A, the Se_{PCR} and Sp_{PCR} for *S. agalactiae* in milk samples were 96.4 and 93.4 %, respectively, whereas the Se_{BC} and Sp_{BC} for *S. agalactiae* were 82.4 and 99.7 %, respectively. For *S. aureus*, in part A, the Se_{PCR} and Sp_{PCR} in milk samples were 87.6 and 98.2 %, respectively, whereas the Se_{BC} and Sp_{BC} were 74.0 and 99.4 %, respectively. Recent studies used LCA in estimating the test characteristics of the PathoProof™ Mastitis PCR Assay and BC in diagnosing IMI caused by *S. agalactiae* and *S. aureus*. At Ct value cutoff ≤ 37, Mahmmod et al. (2013b) reported a Se_{PCR} and Sp_{PCR} for *S. agalactiae* in milk samples of 91.9 and 96.9 %, respectively, and a Se_{BC} and Sp_{BC} of 29.9 and 99.5 %, respectively. Our findings for Se_{PCR}, Sp_{PCR}, and Sp_{BC} fell within the range of those reported by Mahmmod et al. (2013b). The Se_{BC} for *S. agalactiae* in milk samples from our study was higher than the Se_{BC} reported by Mahmmod et al. (2013b). This was despite the use of three different media (calf blood agar, chrome agar, and N-plate agar) compared to the two media (calf blood agar and modified Edward's medium) used in our study. One reason for the inconsistency could then be argued by the difference in incubation: The plates were read only after 24 hours of incubation (Mahmmod et al. 2013b) whereas in our study, the plates were incubated for 48 hours and read after both 24 and 48 hours of incubation. In our study, we experienced that some *S. agalactiae* colonies were not detected until after 48 hours of incubation (data not shown).

The Se_{PCR} and Sp_{PCR} for *S. aureus* at Ct value cutoff ≤ 37 were estimated to 91.2 and 98.6 %, respectively, whereas the Se_{BC} and Sp_{BC} were estimated to 52.8 and 89.4 %, respectively (Mahmmod et al. 2013a). Similar results were reported by Cederlöf et al. (2012) who determined the Se_{PCR} and Sp_{PCR} for *S. aureus* at Ct value cutoff ≤ 37 to 93 and 95 %, respectively, and the Se_{BC} and Sp_{BC} to 94 and 90 %, respectively. Our findings of Se_{PCR} and Sp_{PCR} fell within the range for those reported by Mahmmod et al. (2013a) and Cederlöf et al. (2012) but our Se_{BC} was higher than that estimated by Mahmmod et al. (2013a) but lower than that estimated by Cederlöf et al. (2012) whereas our Sp_{BC} was higher than the Sp_{BC} reported by both studies. The higher Se_{BC} reported by Cederlöf et al. (2012) could be the result of freezing the milk samples until further analysis (Villanueva et al. 1991; Godden et al. 2002). The lower Se_{BC} reported by Mahmmod et al. (2013a) could be the result of different criteria for positivity (criterion of CFU ≥ 2 compared to our criterion of CFU ≥ 1).

It is important to notice that the three studies mentioned (Cederlöf et al. 2012; Mahmmod et al. 2013a; Mahmmod et al. 2013b) all compared PCR on composite milk samples from routine milk recording against BC on aseptic quarter milk samples, and that the study populations differed from our study population. Furthermore, it is important to notice that the PCR assay used in the three

studies (PathoProof™ Mastitis PCR Assay) differed from the PCR assay (Mastit4 qPCR Assay) used in our study. The analytical sensitivity and specificity of the PathoProof™ Mastitis PCR Assay was 100 % in identifying bacteria from isolates originating from bovine mastitis according to Koskinen et al. (2009). Rattenborg et al. (2015) found moderate to high agreement between PathoProof™ Mastitis PCR Assay and Mastit4B qPCR Assay for *S. agalactiae* from BTM samples whereas the agreement was moderate for *S. aureus* (Ct value cutoff ≤ 37). The agreement between PathoProof™ Mastitis PCR Assay and Mastit4B qPCR Assay needs to be investigated further for samples at cow-level in cows with high SCC to clarify whether comparison to results from other studies is accepted. Despite different sample types (composite and quarter milk samples), different study populations, and different PCR tests, it appears that very similar Se_{PCR} and Sp_{PCR} can be observed.

As previously described, the Se_{PCR} and Sp_{PCR} for both *S. agalactiae* and *S. aureus* were similar comparing part A with part B. In addition, Sp_{BC} for both *S. agalactiae* and *S. aureus* were similar comparing the two parts of the study whereas Se_{BC} for especially *S. aureus* differed, ranging from 74.0 % in part A to 57.7 % in part B. Se_{BC} for *S. agalactiae* was 82.4 and 88.6 % in part A and B, respectively. These differences between part A and B could be explained by our selection criteria in part B: Quarters positive with *S. agalactiae* were made a priority over quarters positive with *S. aureus*, and thus agar plates were thoroughly read, especially in search for *S. agalactiae* positive quarters. In part B, we chose not to use the SA*Select*™ medium for identification of *S. aureus* which could be another reason for the lower Se_{BC} for *S. aureus* in part B.

Teat skin samples

Association between Ct values and CFU group

On teat skin, no correlation between Ct values and CFU group was applicable for *S. agalactiae* because of only one BC positive teat skin sample. The correlation for *S. aureus* ranged from -0.13 in part A to -0.31 in part B. When comparing the Ct values with CFU group, some disagreement between PCR and BC was seen. Several samples, especially from teat skin, were PCR negative but BC positive with low numbers of CFU. The reason for this disagreement on teat skin between the two methods could be the difference in the used inoculum which was 100 µl for BC and what corresponds to 10 µl for PCR.

Diagnostic test properties of PCR and BC

Due to a lack of positive cases, results from teat skin samples were not considered for LCA. Instead, among other things, the agreement (kappa) between PCR and BC was determined. For both *S. aureus* and *S. agalactiae*, and both part A and B, the agreement between PCR and BC on teat skin was poor to fair or even worse than by chance, independently of the Ct value cutoff. This poor agreement indicated that PCR and BC detect different 'disease' conditions.

Diagnostic test properties of the PCR test

Teat skin samples were not applicable for LCA because the data could not be split into two populations because of a low number of BC positive findings. Therefore, the diagnostic sensitivity and specificity of the Mastit4 qPCR Assay for the detection of either *S. agalactiae* or *S. aureus* on teat skin were determined using BC as a reference standard. This method has some limitations since several studies have shown lower sensitivity of BC compared to PCR for milk samples (Cederlöf et al 2012; Mahmmod et al. 2013a; Mahmmod et al. 2013b). Whether this is the case for teat skin is uncertain since, to our knowledge, no LCA has been performed on teat skin samples.

The higher Se_{PCR} for *S. agalactiae* compared to *S. aureus* in both part A and B (Table 14) was most probably due to too few observations which was also reflected in the wide CI for *S. agalactiae*: In both part A and B, only a single BC positive sample was found, and this was also positive with PCR (Table 11), hence the Se_{PCR} of 100 % [5.5-100 %]. The low Se_{PCR} for *S. aureus* in part A could be the result of 16 samples positive with BC but negative with PCR (Table 11). One of the reasons for the higher Se_{PCR} for *S. aureus* in part B compared to part A could be that in part B, only one sample was positive with BC but negative with PCR. Another reason could be the sampling procedure which for part B involved rolling of the PCR swab directly on teat skin. When the PCR swab was rolled directly on teat skin, potential bacteria were not diluted in Ringer's solution as in part A. Also, the area from where the PCR swab was taken was larger than for part A. Furthermore, the PCR swab was moistened in a buffer solution which perhaps increased the sensitivity.

Although BC is regarded as the reference standard for identification of mastitis pathogens, it is important to note its limitations as a reference test. If PCR correctly identified bacteria missed with BC, these were defined as false positives, thus underestimating the Sp_{PCR} (and the PPV). In our study, PCR found several positive teat skin samples not identified with BC for both pathogens (Table 11). This could be the reason for the relatively low Sp_{PCR} for both *S. aureus* and *S. agalactiae*. As previously mentioned, in part B the PCR swab was rolled directly on teat skin

which we assume increased the number of samples positive with PCR. But because the majority of samples positive with PCR was still BC negative and thus defined as false positives, the Sp_{PCR} for both pathogens was low.

Differences between PCR and BC

All included analyses in this study showed that PCR and BC have very different properties as diagnostic tests for both milk and teat skin samples. Underlying reasons for the differences in findings could be partly explained by the methods chosen, e.g. difficulties in carrying out standardized teat skin samples under practical conditions, different volumes for BC and PCR analysis, and the choice of plates which also differed from part A to part B. Biological explanations will be discussed in the following.

Milk samples

Regarding differences in test properties of PCR and BC in milk samples, the importance of knowing the underlying disease definition (and at which cutoff) of a specific test to evaluate tests on the same foundation, the limitation of BC regarding the growth of some mastitis pathogens, the variation in bacterial shedding, and the presence of bacterial DNA intramammarily post treatment leading to false positive PCR results are all subjects discussed.

Latent disease definition

In our study, the agreement between PCR and BC improved, expressed as an increase in kappa and decrease in χ^2 values (higher p-values) for McNemar, when lowering the Ct value cutoff (from ≤ 37 to ≤ 32). A similar conclusion was recently reported for *S. agalactiae* and *S. aureus* from milk samples. Both Cederlöf et al. (2012) and Mahmmod et al. (2013b) found that increasing Ct value cutoffs resulted in an increased sensitivity and reduced specificity of PCR whereas for BC the opposite was the case (reduced sensitivity and increased specificity). This implied that changing the Ct value cutoff changed the underlying disease definition, from a truly or heavily infected cow at low Ct value cutoff to simply a positive cow at high Ct value cutoff (Cederlöf et al. 2012; Mahmmod et al. 2013b). Since Ct value cutoff ≤ 37 was the recommended threshold by the manufacturer, this was the only threshold chosen for our LCA. But the above taken into consideration, the choice of Ct value cutoff should depend on the requested disease definition and perhaps also on the pathogen in question.

Growth and shedding of bacteria

The Se_{PCR} was higher than Se_{BC} for both *S. agalactiae* and *S. aureus* in both part A and B. One limitation of BC is that some bacteria are difficult or even impossible to culture (Smith 2009) which could lead to BC false negatives. No growth has been reported from clinical and subclinical mastitis cases ranging from 26.5 and 38.6 % (Bradley et al. 2007) to 32.5 and 55.1 % (Gianneechini et al. 2002), respectively. Thus, the higher Se_{PCR} compared to Se_{BC} in our study was not necessarily the result of several PCR false positives, but could be due to BC false negatives. This is supported by Taponen et al. (2009) who detected mastitis pathogens with PCR in nearly half of the clinical mastitis cases negative with BC. The mastitis pathogens detected were often in substantial quantities (Taponen et al. 2009). Furthermore, if cows are shedding bacteria intermittently or if the bacterial concentration is too low to be detected by culture (i.e. < 100 CFU/ml), the sensitivity of BC decreases (Cai et al. 2003; Steele et al. 2017) which could also explain the higher Se_{PCR} compared to Se_{BC} reported from our study.

PCR, however, can detect low concentrations of bacterial DNA but at the risk of detecting false positives. As previously mentioned, we cannot rule out that some of the farmers had treated the animals reported infected in part A which could explain the proportion of negative samples in part B. According to Wellenberg et al. (2010), bacterial DNA can still be present in the udder after treatment (or self-cure), and the cow can falsely be diagnosed positive with PCR. This can also explain why Se_{PCR} was higher than Se_{BC} in both part A and B. The probability of self-cure or treatment just before sampling is always an important issue to consider, and in these cases, BC might offer a more realistic and reliable picture of the infection status of the cow since no inactivated or dead bacteria are detected.

Teat skin samples

Important aspects regarding differences in test properties of PCR and BC on teat skin samples are discussed in the following section. As for milk samples, the importance of knowing the underlying disease definition is discussed but from a different perspective. In milk samples, the presence of any bacteria, dead or viable, indicates a recent or ongoing infection which both are important information. In teat skin samples, the differentiation between teat skin colonization and teat skin contamination (as a source for IMI) is important why the presence of dead bacteria seems to be less important. PCR as a test for detecting teat skin colonization has some limitations since it cannot distinguish dead and viable bacteria. Other important subjects to discuss regarding different

test properties of PCR and BC include teat disinfectant applied post milking and its effect on teat skin microflora, and CNS as an inhibitor of major udder pathogens.

Disease definition and choice of Ct value cutoff

For teat skin samples, large differences in the number of positive samples according to BC and PCR (Table 11) were seen and furthermore reflected in the χ^2 values (and corresponding p-values) of the McNemar test and kappa coefficients (Table 12). These disagreements question whether the two methods (BC and PCR) detect the same 'disease' condition and if so, what definition of disease condition is suitable. It seems that PCR detects intact bacteria whether they are dead, inactivated, or viable whereas BC detects only viable bacteria.

Viability of bacterial cells

One of the reasons for differences in sensitivity of PCR and BC could be the viability of the detected bacterial cells. Whereas PCR detects intact bacterial cells whether they are viable or nonviable, one of the advantages of BC is the detection of only viable bacterial cells. To determine if samples positive for *S. agalactiae* with PCR really comprised living bacteria, 16 teat skin samples from herd 1 to 3 positive for *S. agalactiae* with PCR and negative with BC were recultured after enrichment step with Todd Hewitt broth. After as little as 24 hours of incubation, both the calf blood agar and the modified Edward's medium were overgrown, thus complicating the reading. The results were inconclusive because all samples were negative, including the positive controls. It is uncertain if refrigerating over several weeks (which unfortunately was the case) could have affected the viability of bacteria.

To investigate this further, all teat skin samples from herd 7 and 8 in part B were cultured after enrichment step with Todd Hewitt broth at the same time as plating without enrichment. Once again, plates were overgrown already after 24 hours of incubation. After receiving PCR results for herd 7 and 8 from DNA Diagnostic A/S, it was clear that the results once again were inconclusive. Samples with known positive status from PCR were negative on plates both with and without enrichment step.

If the samples, in fact, were negative or just impossible to read because of overgrowth is unknown. Jørgensen et al. (2016) and Henriksen (2016) both used the Todd Hewitt broth without the complication of overgrown plates. They used the eSwab (Copan, Italy) in contrast to our rayon swabs, the importance of this difference is unknown.

Teat disinfectant and influence on microflora

In our study, time from milking to sampling of the cow was not known. The application of teat disinfectant post milking changes the microflora on the teat skin. Woodward et al. (1988) found that the re-establishment of the normal flora following teat disinfectant was a prolonged process, and the organisms varied from those present initially. Iodine disinfectant is bactericidal (McDonnell & Russell 1999), but might still leave DNA remnants for PCR detection which could explain the disagreement between BC and PCR findings in our study. If most samplings took place shortly after milking, the teat skin microflora might have been killed due to disinfectant and thus resulting in negative BC results. PCR might, on the other hand, still be able to detect DNA remnants from dead bacteria, resulting in false positive results.

CNS as inhibitor

Another limitation of BC could be the presence of bacteria inhibiting the growth of the bacteria of interest causing low detection rates. CNS isolated from fecal samples from dairy cows have shown inhibitory effects on *in vitro* growth of major udder pathogens as *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* (Wuytack et al. 2017). Woodward et al. (1987) found that approximately 25 % of the isolates of normal teat skin flora were inhibitory *in vitro* to Gram-positive and Gram-negative mastitis pathogens. CNS were present in high numbers on the bovine teat skin in our study, 97 % of the 287 RH quarters investigated were colonized (according to BC) (data not published) which could have inhibited the growth of *S. agalactiae* and *S. aureus* on teat skin and thus contribute to the reason for the different test properties of PCR and BC.

The bovine teat skin as a reservoir

From among the 132 quarters selected for part B, *S. aureus* was isolated from teat skin with BC in 34 quarters whereas *S. agalactiae* was isolated from teat skin with BC in two quarters (Table 9). The difference in number of positive teat skin samples for *S. aureus* and *S. agalactiae* could indicate that *S. aureus* has better adapted to bovine teat skin which is also supported by previously mentioned studies considering bovine teat skin as a reservoir for *S. aureus* (Roberson et al. 1994; Haveri et al. 2008; Piccinini et al. 2009; da Costa et al. 2014). In addition to this, after one to three weeks, *S. aureus* could be isolated from 24 % of the positive quarters whereas *S. agalactiae* could not be isolated from any of the two positive quarters. These cases could point towards *S. aureus* colonization (and a reservoir) rather than contamination, but studies are needed to investigate this further.

Conclusion

S. agalactiae and *S. aureus* could be detected in milk and teat skin samples with both PCR and BC. The number of positive samples detected with BC compared to PCR on teat skin was low, especially for *S. agalactiae*. This can be due to the methods chosen, e.g. sampling with teat skin swabs, which was difficult to standardize under practical conditions, or choice of plates for laboratory work since plates were often overgrown. Other explanations for the low number of positive samples can be of biological character, e.g. inhibition of bacterial growth due to teat disinfectant, CNS as a competing microflora, or the ability of *S. aureus* and *S. agalactiae* to colonize the teat skin.

The agreement between BC and PCR for the detection of *S. agalactiae* and *S. aureus* on teat skin was poor, thereby indicating that the two methods do not measure the same disease condition. It is very likely that PCR detected viable as well as dead and inactivated bacteria whereas BC only detected viable bacteria. There were too few positive samples from teat skin to compare Ct values and CFU statistically. Further studies are needed to evaluate the importance of the teat skin as a reservoir before introducing teat skin swabs as a diagnostic tool in herd health management.

Se_{PCR} was higher than Se_{BC} for *S. aureus* and *S. agalactiae* in milk samples in both part A and B. The differences in test properties of PCR and BC can be explained by different factors, e.g. variation in bacterial shedding, the underlying disease definition that changes at different cutoffs, the presence of bacterial DNA intramammarily post treatment, and limitations of BC due to differences in bacterial growth and laboratory methods chosen. There was a strong correlation between Ct values and CFU groups for *S. aureus* and *S. agalactiae*, indicating that the higher the number of CFU in the sample, the lower the Ct value. In the light of the results of this study, PCR swabs seem to be a more sensitive method for diagnosing IMI with *S. agalactiae* or *S. aureus* compared to BC.

Perspectives

To evaluate the importance of the teat skin as a reservoir for *S. agalactiae* and *S. aureus*, this study has shown that other methods are needed for the detection of *S. agalactiae* on teat skin since only two positive samples were detected with BC. Regarding laboratory work, the inclusion of dilution series to overcome overgrown plates has previously been mentioned as a possibility. Another suggestion would be to include chrome agar plates in the protocol to increase the sensitivity of BC. Mahmmod et al. (2013b) included chrome agar in their study, but since this is the first study investigating the presence of *S. agalactiae* on teat skin, the effect is unknown.

Further studies on the reservoirs of *S. agalactiae* are needed. If the teat skin or farm environment really comprise important reservoirs for IMI with *S. agalactiae,* then eradication based on either BTM samples or quarter samples might not be reliable. Furthermore, if *S. agalactiae* in the future will be regarded as opportunistic instead of contagious, then perhaps eradication does not seem reasonably. In any circumstances, the control strategies for *S. agalactiae* should be rewritten.

The discussion about viability of the detected bacterial cells could have been avoided if the PCR was able to distinguish dead and viable bacteria. For further studies comparing PCR to BC on especially teat skin, RT-PCR, where analysis is performed on RNA, is recommended. To further investigate the disagreement between BC and PCR in our study, fresh Ringer's solution could have been applied to inoculated plates. From this Ringer's solution, the swab for PCR analysis could have been taken. According to Krömker (2017, personal communication), only viable bacterial cells are present in the Ringer's solution why with this method, the PCR result would only reflect viable bacteria. In this way, PCR and BC can be compared on the same foundation.

To evaluate the dynamics of mastitis and its influence on diagnostics, a further investigation based on the data collected in the present study could be interesting. For example, whether milk samples positive with PCR but negative with BC in part A were positive with both methods in part B, indicating that PCR detected the infection before BC. To evaluate the importance of the teat skin as a reservoir and to discriminate between colonization and contamination, it could also be interesting to investigate the association between the presence of *S. agalactiae* and *S. aureus* on teat skin and in milk over time, i.e. whether quarters positive on teat skin in part A have IMI in part B.

All data from the AMS for the sampled cows in part A are available. It would be interesting to investigate the association between the pathogens present on the teat skin and the time from milking. In continuation of this, the presence of bacteria on the teat skin could be compared to the degree of coverage by the teat disinfection. If more cows are colonized (or the number of CFU is higher) just after milking compared to several hours after milking, this could support that the cows are infected during milking (as expected for contagious mastitis pathogens), and that teat disinfectant is ineffective. If the opposite is true i.e. more cows are colonized several hours after milking, this could either support the idea of environmental reservoirs or indicate a satisfactory effect of teat disinfection just after milking.

Another interesting investigation could be the determination of strain types found in our study. Some studies indicate that human strain types might comprise an important source of the continuously high prevalence of *S. agalactiae* (Zadoks et al. 2011; Lyhs et al. 2016). A survey of 111 isolates from BTM conducted in Denmark in 2009 showed that the most common strains isolated were sequence type (ST) 1 (28 %) and ST23 (23 %) which are sequence types previously primarily associated with human infection (unpublished data, cf. Zadoks et al. 2011). Another interesting aspect of strain typing the isolates from our study would be whether the same strain types are found in the same herd, indicating a contagious behavior, or if several types are found (Zadoks et al. 2011).

This study has shown that the use of teat skin swabs needs further investigation before introducing as part of herd health management whereas the use of PCR swabs on milk samples seems to be a more sensitive solution compared to BC. This is important, especially when dealing with bacteria such as *S. agalactiae*, where a high diagnostic sensitivity is required not to overlook any present bacteria as part of eradication. When taking milk samples for PCR analysis, aseptically taken samples are decisive for a reliable result. Other important considerations before sampling include cyclic shedding of bacteria and recent treatment which both might influence test results.

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Appendix

Appendix I: Modified Edward's medium formula

Produced at DTU Vet, National Veterinary Institute, Frederiksberg, Denmark with inspiration from Jørgensen et al. (2011).

Modified Edward's medium plates (Oxoid, Thermo Scientific^{TM}) supplemented with 5 % calf blood and 2 % filtrate of a b-toxin producing *S. aureus*. This agar is selective for streptococci and shows the CAMP reaction on primary isolation.

To produce the *S. aureus* filtrate, a b-toxin producing *S. aureus* strain was grown at 37°C for 5 days in 100 ml Brain Heart Infusion broth (Becton Dickinson, Sparks, MD) in a 250 ml Erlenmeyer flask. The culture was filtered through a grade 595 1/2 folded qualitative filter paper (WhatmanTM GE Healthcare Life, Buckinghamshire, UK). Aliquots of 20 ml filtrate were frozen in 50 ml Falcon tubes (CELLSTAR1TUBES, Greiner Bio-one, Kremsmünster, Austria) that were thawed at room temperature (20–22°C) before use.

Modified Edward's medium plates were prepared according to the manufacturer's instructions, but when the agar had cooled to 45–50°C, 5 % calf blood and 2 % of the b-toxin producing *S. aureus* filtrate were added before the plates were poured. Each batch of Edward's medium supplemented with *S. aureus* filtrate was checked for CAMP reaction using identified *S. agalactiae* strain as 'positive control'.

Appendix II: Protocol – DNA Diagnostic FLOQSwabs

Sample collection:

1. DRY SURFACE: Remove the swab from the tube and moderately wet the tip with **Swab Buffer V1** (do not saturate the swab tip). Swab across the dry surface.
   WET SURFACE: Remove the swab from tube and swab across the wet surface. Try not to oversaturate the tip (avoid dripping of sample in tube).
   LIQUID SAMPLE: Remove the swab from tube and dip the tip in the sample, but do not oversaturate the tip (avoid dripping of sample in tube).
2. Insert the swab back into the tube. Make sure that the lid is properly closed. Mark/write on the tube with for sample identification.
3. Store and send the swab in tube at ambient temperature. The sample will dry out on the swab tip, due to a desiccant in the swab lid.

Sample elution and DNA extraction:

4. Add **750uL Swab Elution Buffer** in a 2mL deepwell (or 2mL tube).
5. Remove the swab from the tube. If using deepwell plates, also remove the swab tip and plastic stick from the swab lid. Insert the swab tip into the deepwell (or 2mL tube) containing Swab Elution Buffer.
6. Incubate 10 – 120 minutes at 37 °C.
7. Stir deepwell plate with swabs on 96-well vortexer at 500 rpm for 2 minutes. Make sure that the liquid does not spill out of the wells.
   If using single tubes, stir the swab around in the liquid and try to press the swab against the well wall to press out liquid.
8. Remove and discard the swab.

From this step, the protocol is the same as the DNA Diagnostic Mastit4 'DNA purification' protocol (step 3 and on).

9. Spin Deep Well Plate (or 2mL tubes) at 5000xg for 5 min. Remove supernatants from the top with tips connected to a vacuum system. Be careful not to touch or remove the bacterial pellets.
10. Add 1 mL Wash Buffer to each well/tube. Cover with sealing tape/close tube.
11. Spin at 5000xg for 5 min. Remove supernatants from the top with tips connected to a vacuum system. Be careful not to touch or remove the bacterial pellets. It is important to remove the supernatant completely.
12. Prepare fresh Lysis-I Mix by adding 6 µL *Mix I additive* to 54 µL *Lysis buffer-I* and mix.

Lysis-I Mix	1 reaction	9 reaction	100 reactions
Lysis Buffer-I	54 µL	486 µL	5400 µL
Mix I additive	6 µL	54 µL	600 µL
TOTAL	60 µL	540 µL	6000 µL

13. Add 60 µL Lysis-I Mix, to each pellet and cover with sealing tape (or close tube). Vortex the Deep Well plate/tube at least 10 seconds, or continue until pellet is homogenized (for some samples, pipetting up-and-down might be necessary). Spin 20 seconds at 1000xg to bring Lysis mix to the tube bottoms.
14. Remove caps from 96 well plate with clear 0.2 mL tubes.
15. Use an 8-channel pipette with filter tips for transfer of 60 µL from each well of the Deep Well Plate into the corresponding tubes in the 96 well plate (clear 0.2 mL). Close tubes with the caps. Avoid cross contamination. Use one new tip per tube.
16. Incubate the 96 well plate at 37°C for 20 min.
17. Incubate the 96 well plate at 95°C for 15 min.
18. Cool the 96 well plate on ice for 5 min

    Note: The incubation at 37°C and 95°C can be done using a PCR instrument programmed: 37°C for 20 min. → 95°C for 15 min. → 4°C for 5 min.
19. Centrifuge the 96 well plate/tube at 5000xg for 5 min. at room temperature. Note it is important to use a 96 tube support for the 96 well plate during centrifugation.
20. Carefully remove the caps from the 96 well plate/tubes. Use an 8-channel pipette with filter tips to transfer 5 µL of each aqueous phase directly to the corresponding tubes of the 96 well plate with qPCR Master Mix in step 24.
21. The remaining purified DNA can be stored at -20°C for long time storage.

qPCR analysis

22. Take a 96 Well Plate with qPCR Master Mix (DNA Diagnostic qPCR master mixes only – E.g. Mastit4, TBC4, Salmonella Velox, ect.) from -20°C, place on ice for five minutes and spin 20 seconds at 1000xg to bring qPCR Master Mix to the tube bottoms.
23. Discard the seal from the 96 Well Plate with qPCR Master Mix and place the 96 well plate on ice.
24. Transfer 5 µL purified DNA from step 20 to each of the corresponding tubes in the 96 well plate containing qPCR Master Mix.
25. Carefully close the qPCR tubes with a new optical lid. Spin the tubes briefly before transfer of the 96 Well Plate qPCR reactions to the qPCR instrument. Note it is important to keep the optical lids clean.

    Note: It is important to keep Master Mix on ice while loading template.
    Please refer to the protocol of the selected DNA Diagnostic qPCR kit for further instructions.

Received from DNA Diagnostic A/S [July 11, 2017]

Appendix III: Agreement between calf blood agar and selective medium

Part A

Agreement between modified Edward's medium (Edwards) and calf blood agar (Blood)

BC Milk *S. agalactiae*	Edwards 1	Edwards 0	Total
Blood 1	18	1	19
Blood 0	0	268	268
Total	18	269	287

BC Teat *S. agalactiae*	Edwards 1	Edwards 0	Total
Blood 1	1	0	1
Blood 0	0	286	286
Total	1	286	287

Agreement between SA*Select*™ (SA Select) and calf blood agar (Blood)

BC Milk *S. aureus*	SA Select 1	SA Select 0	Total
Blood 1	16	0	16
Blood 0	5	261	266
Total	21	261	282

BC Teat *S. aureus*	SA Select 1	SA Select 0	Total
Blood 1	13	5	18
Blood 0	5	264	269
Total	18	269	287

Part B

Agreement between modified Edward's medium (Edwards) and calf blood agar (Blood)

BC Milk *S. agalactiae*	Edwards 1	Edwards 0	Total
Blood 1	24	1	25
Blood 0	4	103	107
Total	28	104	132

BC Teat *S. agalactiae*	Edwards 1	Edwards 0	Total
Blood 1	0	1	1
Blood 0	0	131	131
Total	0	132	132

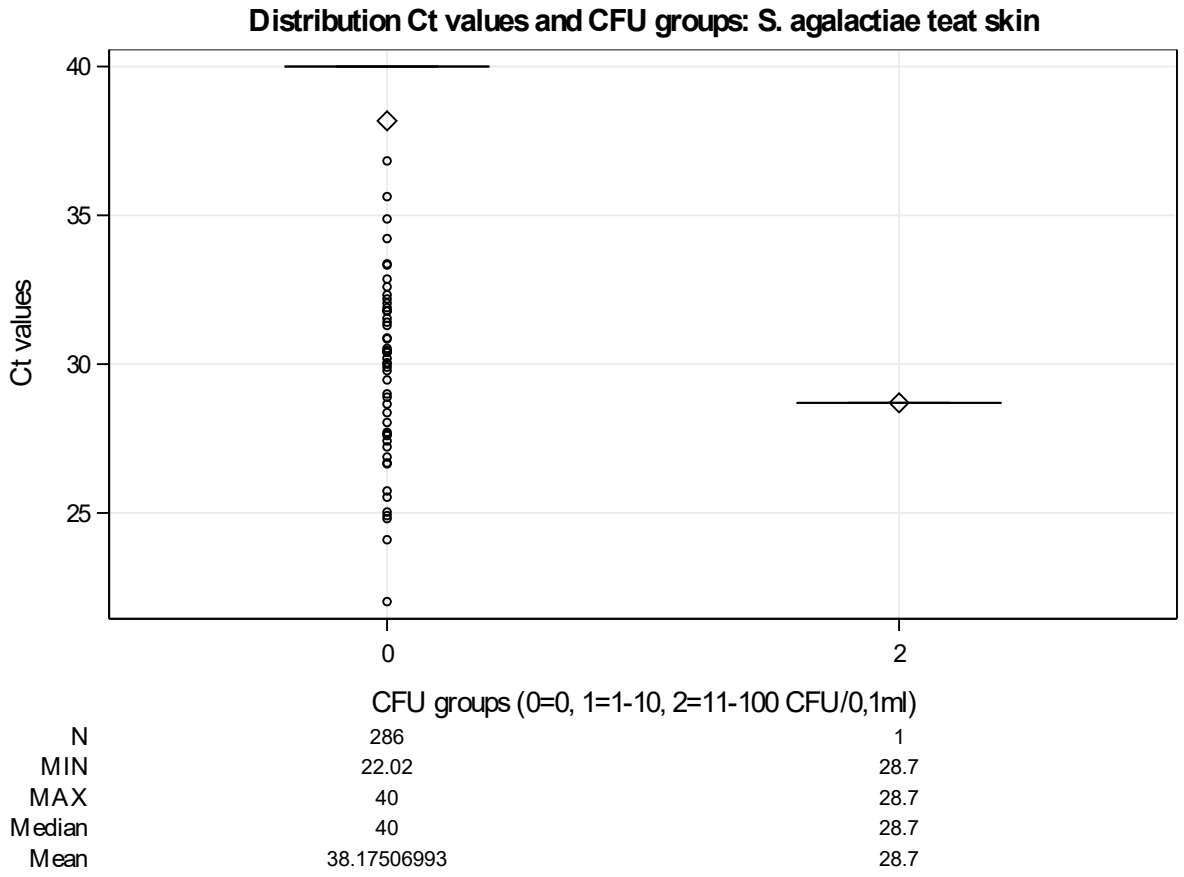
BC: Bacteriological culture

1: Positive on the given growth medium

0: Negative on the given growth medium

Appendix IV: Box-and-whisker plots for *S. agalactiae* on teat skin

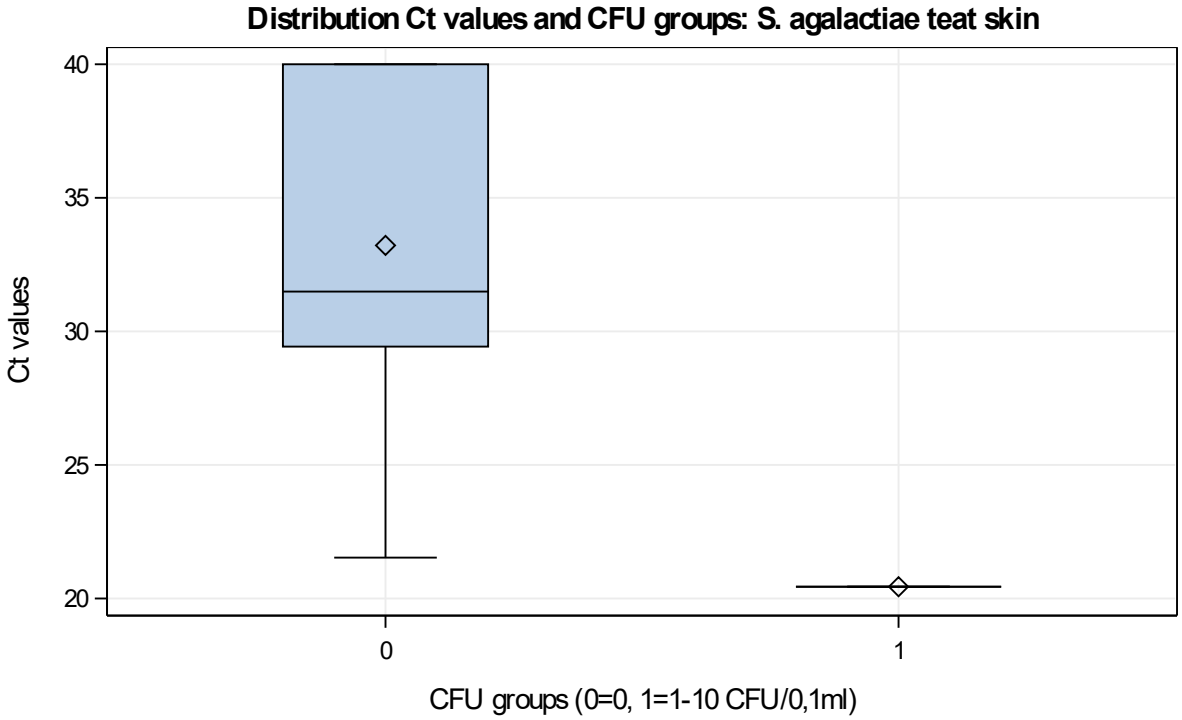
Part A



	0	2
N	286	1
MIN	22.02	28.7
MAX	40	28.7
Median	40	28.7
Mean	38.17506993	28.7

Figure A The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. agalactiae* in teat skin samples from part A. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean.

Part B



	0	1
N	131	1
MIN	21.53	20.44
MAX	40	20.44
Median	31.49	20.44
Mean	33.218549618	20.44

Figure B The distribution of cycle threshold (Ct) values in relation to colony forming units (CFU) group for *S. agalactiae* in teat skin samples from part B. Descriptive statistics are showed, including number (N) of observations, minimum value (MIN), maximum value (MAX), median and mean.

Appendix V: Positive and negative samples in relation to sampling procedure

		BC first			**PCR first**		
		PCR 1	PCR 0	Total	PCR 1	PCR 0	Total
Teat	BC 1	0	0	0	1	0	1
S. agalactiae	BC 0	44	20	64	40	27	67
	Total	44	20	64	41	27	68
Teat	BC 1	5	1	6	9	0	9
S. aureus	BC 0	37	21	58	34	25	59
	Total	42	22	64	43	25	68

Table A The distribution of positive and negative samples in relation to sampling procedure (bacteriological culture (BC) first or polymerase chain reaction (PCR) first). 1: Positive with the given method, 0: Negative with the given method.

Appendix VI: Latent class analysis estimates

Cycle threshold (Ct) value cutoff ≤ 37

Population split: Automatic milking system (AMS) type

Test1= polymerase chain reaction (PCR), Test2 = bacteriological culture (BC)

Milk

Part A

1- *S. agalactiae*

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
p[1]	0.07363	0.03108	3.347E-4	0.03093	0.06662	0.1504	10001	60000
p[2]	0.1393	0.04108	1.805E-4	0.0695	0.1359	0.2291	10001	60000
se[1]	0.9495	0.04837	3.477E-4	0.8201	0.9639	0.9987	10001	60000
se[2]	0.7876	0.1616	0.001789	0.4361	0.8244	0.9933	10001	60000
sp[1]	0.9362	0.02495	2.554E-4	0.8921	0.9337	0.9919	10001	60000
sp[2]	0.9959	0.004032	2.897E-5	0.9852	0.9972	0.9999	10001	60000

2- *S. aureus*

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
p[1]	0.05092	0.01785	9.544E-5	0.02183	0.04895	0.09064	10001	60000
p[2]	0.2538	0.05712	2.828E-4	0.1499	0.251	0.3723	10001	60000
se[1]	0.8656	0.08045	4.245E-4	0.6824	0.8761	0.9879	10001	60000
se[2]	0.7394	0.1131	7.149E-4	0.5176	0.7401	0.9556	10001	60000
sp[1]	0.9805	0.01287	8.599E-5	0.951	0.9824	0.9989	10001	60000
sp[2]	0.9921	0.006697	4.202E-5	0.9749	0.9938	0.9997	10001	60000

Part B

1- *S. agalactiae*

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
p[1]	0.1602	0.04568	2.924E-4	0.08298	0.1559	0.261	10001	60000
p[2]	0.462	0.08495	3.914E-4	0.2994	0.4607	0.6308	10001	60000
se[1]	0.9418	0.04943	3.223E-4	0.8173	0.9544	0.9981	10001	60000
se[2]	0.8695	0.09299	6.981E-4	0.657	0.8857	0.9946	10001	60000
sp[1]	0.9135	0.03806	2.514E-4	0.8361	0.9143	0.9859	10001	60000
sp[2]	0.9752	0.0179	9.974E-5	0.9315	0.9787	0.9986	10001	60000

2- *S. aureus*

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
p[1]	0.05088	0.02981	1.916E-4	0.009277	0.04534	0.1223	10001	60000
p[2]	0.3867	0.09537	5.27E-4	0.2092	0.3838	0.581	10001	60000
se[1]	0.9072	0.08482	6.124E-4	0.6826	0.9318	0.9972	10001	60000
se[2]	0.5822	0.1481	9.066E-4	0.3108	0.5765	0.8865	10001	60000
sp[1]	0.9445	0.03024	1.928E-4	0.8781	0.9472	0.9945	10001	60000
sp[2]	0.9908	0.0091	6.302E-5	0.9664	0.9935	0.9998	10001	60000

Appendix VII: Prevalence in part A

Sample type	Pathogen	Method	Prevalence (Part A)
Milk	*S. agalactiae*	BC	6.6 %
		PCR	13.9 %
	S. aureus	BC	7.7 %
		PCR	10.1 %
Teat	*S. agalactiae*	BC	0.3 %
		PCR	18.1 %
	S. aureus	BC	8.0 %
		PCR	15.7 %

Table B The prevalence of positive right hind (RH) quarters in part A according to method (bacteriological culture (BC) or polymerase chain reaction (PCR)) among cows with high (> 200.000 cells/ml) somatic cell count (SCC) in herds with positive status for *S. agalactiae*. Cycle threshold (Ct) value cutoff < 40 and colony forming units (CFU) ≥ 1.