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Diagnosis of *Staphylococcus aureus* and *Streptococcus agalactiae* mastitis

Taking into account infection dynamics and teat skin as a reservoir

PhD Thesis 2018 • Line Svennesen

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Principal supervisor: Professor Søren Saxmose Nielsen
Department of Veterinary and Animal Sciences,
University of Copenhagen,
Denmark

Previously:
Associate professor Ilka Christine Klaas
Department of Veterinary and Animal Sciences,
University of Copenhagen,
Denmark

Co-supervisor: Dr. Karl Pedersen
Department of Animal Health and Antimicrobial Strategies,
National Veterinary Institute,
Sweden

Previously:
Dr. Torben Werner Bennedsgaard
Department of Animal Science,
Aarhus University,
Denmark

Assessment Committee: Professor Hans Houe (Chairman)
Department of Veterinary and Animal Sciences,
University of Copenhagen,
Denmark

Professor Ruth N. Zadoks
Institute of Biodiversity, Animal Health and Comparative Medicine,
College of Medical, Veterinary and Life Sciences,
Great Britain

Dr. Morten Dam Rasmussen
Department of Engineering,
Aarhus University,
Denmark

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F. Troels Ronco, Ilka C. Klaas, Marc Stegger, Line Svennesen, Lærke B. Astrup, Michael Farre and Karl Pedersen (2018). **Genomic investigation of *Staphylococcus aureus* isolates from bulk tank milk and dairy cows with clinical mastitis.** In *Journal of Veterinary Microbiology*, 2018. https://doi.org/10.1016/j.vetmic.2018.01.003

G. Yasser S. Mahmmod, Bettina Nonnemann, Line Svennesen, Karl Pedersen and Ilka C. Klaas (2018). **Typeability of MALDI-TOF assay for identification of non-aureus staphylococci associated with bovine intramammary infections and teat apex colonization.** In *Journal of Dairy Science*, 2018. https://doi.org/10.3168/jds.2018-14579

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I. Line Svennesen, Yasser Mahmmod, Karl Pedersen, Volker Krömker and Ilka C. Klaas (2017). **Association between teat skin colonization and intramammary infections with *Staphylococcus aureus* and *Streptococcus agalactiae*.** In proceedings of Copenhagen Cattle Seminar 2017, Copenhagen, DK. *Abstract and oral presentation*

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K. Line Svennesen, Torben W. Bennedsgaard, Karl Pedersen and Ilka C. Klaas (2016). **Short time variation in daily shedding of contagious mastitis pathogens.** In proceedings of Copenhagen Cattle Seminar 2016, Copenhagen, DK. *Abstract and oral presentation*

L. Line Svennesen, Torben W. Bennedsgaard, Karl Pedersen and Ilka C. Klaas (2016). **Short time variation in daily shedding of *Streptococcus agalactiae* and *Staphylococcus aureus* determined by bacterial culture and PCR test.** In Proceedings of International Dairy Federation International Mastitis Conference 2016, Nantes, France. *Abstract and poster*

Summary

Staphylococcus aureus and *Streptococcus agalactiae* are udder pathogens that cause mastitis or intramammary infections (IMI) in dairy cattle worldwide. Mastitis is the most common disease related to milk production and decreases milk yield and milk quality, leading to economic losses as well as increased antibiotic consumption and reduced animal welfare. Despite focus on control, the apparent herd prevalence of *Staph. aureus* is high and the proportion of *Strep. agalactiae*-infected herds is increasing in the Scandinavian countries.

Staph. aureus and *Strep. agalactiae* are considered contagious udder pathogens, with the milk from infected udder quarters as the main reservoir of bacteria. This also means that spread is thought to occur from cow to cow and primarily during milking, but other reservoirs might exist since the control of *Staph. aureus* and *Strep. agalactiae* appears to be difficult. Furthermore, uncertainty in the interpretation of diagnostic test results may influence the success of control programmes implemented for these pathogens.

The overall aim of this PhD project was to gain more knowledge about the dynamics of *Staph. aureus* and *Strep. agalactiae* IMI and about the teat skin as a reservoir for these contagious udder pathogens. With this knowledge, it would be possible to improve recommendations for the efficient diagnosis and control of contagious mastitis in dairy herds.

Data were collected in two sampling activities. Sampling activity A included collection of quarter-level teat skin and milk samples from 300 cows with high somatic cell count (SCC) from eight herds positive for *Strep. agalactiae*. Teat skin and milk samples from all quarters were cultured and samples from one quarter per cow were analysed with polymerase chain reaction (PCR). In Sampling activity B, 24 quarters with *Staph. aureus* and 16 quarters with *Strep. agalactiae* were investigated over 21 days. The quarters were naturally infected and selected based on a positive PCR test. Daily milk samples from each of the investigated quarters were analysed using bacterial culture (BC), PCR and SCC.

In the first study, data from Sampling activity A were used to investigate the association between teat skin colonisation and IMI with *Staph. aureus* or *Strep. agalactiae*. This was done using logistic regression models separately for BC and PCR results. The results showed that teat skin colonisation with *Staph. aureus* (detected by BC) was associated with *Staph. aureus* IMI in the same quarter, as the odds of having an IMI was higher when the pathogen was detected on teat skin. However, no association between teat skin colonisation and IMI was found when using PCR. This finding raised the question of teat skin colonisation versus contamination, as the PCR test has the ability to detect lower concentrations than the applied BC method, as well as the ability to detect inactivated bacteria. Therefore, the clinical relevance of *Staph. aureus* detected by PCR on teat skin should be further investigated. The proportion of quarters positive for *Strep. agalactiae* on teat skin was low when using BC, indicating that BC is a poor method for detecting *Strep. agalactiae* in non-milk samples, or that *Strep. agalactiae* does not colonise teat skin. In contrast, using PCR resulted in a higher proportion of teat skin samples positive for *Strep. agalactiae*, and the odds of IMI detected by PCR was higher when *Strep. agalactiae* was also detected on teat skin (using PCR). In conclusion, teat skin colonisation or contamination could be a risk factor for IMI,

indicating that the teat skin should be considered as a potential reservoir and teat skin hygiene recommended in order to reduce transmission in relation to the control of *Staph. aureus* and *Strep. agalactiae*.

The test accuracy of BC and PCR for teat skin and milk samples was evaluated in the second study. Data from Sampling activity A (where BC and PCR results were available from the same quarters) were analysed in a latent class analysis (LCA). The results showed that the PCR test should be the preferred test in terms of both sensitivity (Se) and specificity (Sp) for the detection of *Staph. aureus* in teat skin samples and aseptically collected quarter milk samples. For the detection of *Strep. agalactiae,* the PCR test is preferable in terms of Se, but BC had the highest Sp. Generally, the Se of BC was low (around 50%) for both *Staph. aureus* and *Strep. agalactiae* in milk and teat skin samples.

In the third study, diagnostic test patterns of 40 quarters were investigated in three steps: firstly, the daily test results from Sampling activity B were used to create profiles for each of the quarters investigated. Secondly, 30 mastitis experts were asked to group and establish diagnoses for the quarters. Lastly, the experts' diagnoses were used as a reference for the daily infection status when estimating test performance for each of the three tests (BC, PCR and SCC). It appeared from the diagnostic test patterns that consistent patterns (diagnosed as persistent infections) were mostly found. For *Staph. aureus,* varying patterns were also recognised and diagnosed as dynamic infections. For *Strep. agalactiae,* there was a group of quarters for which it appeared to be difficult to assign a diagnosis. This was shown by disagreement among the experts' interpretations and diagnoses, indicating that either mastitis terminology is inconsistent or the guidelines for diagnosis require improvement. In addition, a number of quarters were diagnosed as non-infected despite a positive screening test, indicating the possibility of false-positive test results, and meaning that quarters may be falsely diagnosed as infected based on a single positive PCR sample. In addition to persistent infections, dynamic infections and healthy quarters, the following diagnoses were given by the experts: persistent infections with false-negative test results, new infections, new infections with false-negative test results, transient infections, resolving infections and healthy quarters with false-positive test results. This suggests that there may be biological factors and courses of infection that should be taken into consideration alongside the diagnostic test performance when interpreting test results. The estimated test performance of BC and PCR was generally high when the expert diagnoses were used to set the reference of infection, whereas the Se and Sp of SCC were lower, indicating that the SCC appeared to be of minor importance to the experts when diagnosing IMI. Furthermore, it appeared from some of the diagnoses that a lower Sp was acceptable when Se increased, which could be because the experts preferred a high Se when diagnosing contagious and major pathogens like *Staph. aureus* and *Strep. agalactiae*.

In conclusion, the results of this PhD project have contributed to a better understanding of the diagnostic test results of *Staph. aureus* and *Strep. agalactiae,* including the infection dynamics and teat skin as a reservoir. The results have been used to suggest some recommendations for the interpretation of diagnostic test results based on different sampling and test methods. It is of particular importance to consider the goal of testing and thereby decide whether high Se or Sp is needed. The low Se of BC based on the LCA makes the PCR test preferable when high Se is

required. However, the ability of the PCR test to detect non-viable and low concentrations of bacteria may increase the risk of getting positive test results with no clinical relevance, and therefore BC would be preferred if high Sp is needed. The teat skin should be considered as a reservoir particularly in the interpretation of test results from non-aseptically collected samples. To assess IMI, SCC should be used as a supplementary test to BC or PCR. In general, repeated testing is needed if the course of infection should be taken into account and if the risk of giving a false diagnosis should be minimised.

Sammendrag

Staphylococcus aureus og *Streptococcus agalactiae* er bakterier, der globalt set forårsager yverbetændelse (mastitis) eller intrammamære infektioner (IMI) hos malkekvæg. Mastitis er den hyppigst forekommende sygdom i mælkeproduktionen og forårsager nedsat mælkeydelse og nedsat mælkekvalitet. Derved fører masitits til økonomiske produktionstab, øget forbrug af antibiotika og nedsat dyrevelfærd. Selvom der gennem mange år har været fokus på kontrol af mastitis med *Staph. aureus* og *Strep. agalactiae*, er forekomsten på besætningsniveau fortsat høj for *Staph. aureus* og stigende for *Strep. agalactiae* i de skandinaviske lande.

Staph. aureus og *Strep. agalactiae* betegnes begge som smitsomme yverpatogener, der primært smitter mellem køer via mælk fra inficerede mælkekirtler. Derfor betragtes malkning som den primære risiko for smitte, men der kan dog være tale om andre smittekilder, idet kontrol med *Staph. aureus* og *Strep. agalactiae* kan være problematisk. Usikkerhed ved tolkning af diagnostiske testresultater kan desuden have indflydelse på, hvorvidt der opnås succes med implementering af eventuelle saneringsprogrammer.

Formålet med dette ph.d.-projekt var at opnå mere viden om dynamikken af IMI med *Staph. aureus* og *Strep. agalactiae* og at undersøge pattehuden som et reservoir for bakterierne, med henblik på at forbedre anbefalingerne for diagnostik og kontrol af smitsom mastitis i malkekvægsbesætninger.

Der blev i projektet indsamlet data i to aktiviteter. Dataindsamling A blev foretaget i 8 *Strep. agalactiae*-positive besætninger og bestod af pattehuds- og mælkeprøver på kirtelniveau fra 300 køer med højt celletal. Pattehuds- og mælkeprøver fra alle kirtler blev undersøgt ved bakteriologisk dyrkning (BU) og prøver fra én kirtel per ko blev desuden analyseret med polymerase chain reaction (PCR) test. Ved dataindsamling B blev der, baseret på en indledende positiv PCR test, udvalgt 24 kirtler med *Staph. aureus* og 16 kirtler med *Strep. agalactiae*. Alle kirtler blev fulgt i 21 dage, og en daglig mælkeprøve fra hver kirtel blev analyseret med BU, PCR og SCC.

I det første studium blev sammenhængen mellem forekomst af *Staph. aureus* eller *Strep. agalactiae* på pattehud og i mælk (IMI) fra samme kirtel undersøgt. BU og PCR resultater fra dataindsamling A blev hver for sig analyseret i en logistisk regressionsmodel. Resultaterne viste, at *Staph. aureus* på pattehuden (påvist med BU) var associeret med *Staph. aureus* IMI i samme kirtel, og odds for at have IMI var højere ved fund af *Staph. aureus* på pattehuden. Denne association kunne dog ikke genfindes ved brug af PCR testen, hvilket rejste et spørgsmål om påvisning af hhv. pattehudskolonisering eller -kontaminering; PCR testen har en lavere detektionsgrænse end BU og har tilmed evnen til at påvise ikke-levedygtige bakterier, hvorfor den kliniske betydning af fund af *Staph. aureus* på pattehud med PCR testen bør undersøges nærmere. Andelen af pattehudsprøver, hvorfra det var muligt at dyrke *Strep. agalactiae*, var lav, hvilket kan skyldes, at BU er en uegnet metode til at genfinde *Strep. agalactiae* i andre prøver end mælk, eller at *Strep. agalactiae* ikke koloniserer pattehuden. Ved brug af PCR fandtes der dog en højere andel af positive pattehudsprøver og tilmed fandtes højere odds for IMI med *Strep. agalactiae*, hvis pattehuden var PCR positiv. Resultaterne antydede, at pattehudskolonisering eller kontaminering

kan være en risikofaktor for IMI, hvorfor pattehud bør overvejes som et potentielt reservoir og hygiejne i forbindelse med pattehuden kan anbefales ved saneringstiltag mod *Staph. aureus* og *Strep. agalactiae*.

I det andet studium blev BU og PCR evalueret som diagnostiske metoder til påvisning af *Staph. aureus* og *Strep. agalactiae* i pattehuds- og mælkeprøver. Testresultater fra kirtler, der var undersøgt med både BU og PCR ved dataindsamling A, blev analyseret i en latentklasseanalyse. Resultaterne viste, at PCR testen bør være førstevalg både i forhold til sensitivitet (Se) og specificitet (Sp) til påvisning af *Staph. aureus* i mælk og på pattehud, når prøverne er udtaget aseptisk. PCR testens Se til påvisning af *Strep. agalactiae* var også højere end BU, hvorimod BU havde en højere Sp. Generelt var Se for BU lav (omkring 50 %) for både *Staph. aureus* og *Strep. agalactiae* i pattehuds- og mælkeprøver.

I det tredje studium blev de diagnostiske testmønstre undersøgt for de 40 kirtler med enten *Staph. aureus* eller *Strep. agalactiae*. Dette inkluderede tre trin; 1) profiler for hver kirtel blev dannet ud fra de daglige testresultater i dataindsamling B, 2) profilerne blev grupperet og givet en diagnose af 30 mastitis-eksperter, 3) den diagnostiske testakkuratesse for BU, PCR og SCC blev evalueret med eksperternes diagnoser som reference for den daglige infektionsstatus for hver kirtel. Hovedparten af de diagnostiske testmønstre var stabile og blev af eksperterne diagnosticeret som persisterende infektioner. For *Staph. aureus* fandtes også mere varierende mønstre, diagnosticeret som dynamiske infektioner. For *Strep. agalactiae* var der en gruppe af kirtler, som tilsyneladende var svære at diagnosticere, eftersom eksperterne var uenige om diagnosen. Dette kan skyldes, at mastitisterminologien er for dårligt defineret, eller at vejledninger til at stille diagnoser kan forbedres. Et antal kirtler blev også diagnosticeret som ikke-inficerede, til trods for den positive screeningstest. Dette betyder at falskpositive testresultater kan forekomme og derved resultere i en forkert diagnose hvis den baseres på et enkelt positivt PCR testresultat. Ud over persisterende infektioner, dynamiske infektioner og raske (ikke-inficerede) kirtler, fandtes også diagnoserne: persisterende infektioner med falsknegative testresultater, nyinfektioner, nyinfektion med falsknegative testresultater, forbigående infektioner, infektioner under helbredelse, og raske kirtler med falskpositive testresultater. Alle disse forskellige diagnoser indikerer, at der ud over den diagnostiske test akkuratesse også er biologiske faktorer og retninger for infektion, som skal overvejes ved tolkning af testresultater. Generelt var den estimerede testakkuratesse høj for BU og PCR ved brug af eksperternes diagnoser som reference for infektionsstatus. Se og Sp for SCC var lavere, hvilket indikerer, at SCC blev tillagt mindre betydning af eksperterne ved deres diagnosticering af IMI. Desuden viste nogle af de givne diagnoser at en lavere Sp var acceptabel, hvis det var på bekostning af en høj Se. Dette kan skyldes, at eksperterne prioriterede en høj Se til diagnosticering af *Staph. aureus* og *Strep. agalactiae*, der repræsenterer de væsentlige smitsomme mastitispatogener.

Resultaterne af overnævnte studier har bidraget til en bedre forståelse for diagnostiske testresultater for *Staph. aureus* og *Strep. agalactiae*, samt for infektionsdynamikken og pattehuden som reservoir for de to patogener. Resultaterne er til sidst brugt som grundlag for en række anbefalinger vedrørende tolkning af diagnostiske testresultater. Forud for valg af testmetode er

det vigtigt at overveje målet med at teste og derved beslutte, om en høj Se eller en høj Sp er vigtigst. Den lave Se for BU fundet ved latentklasseanalysen gør, at PCR bør vælges, hvis en høj Se ønskes. På den anden side kan PCR testens lave detektionsgrænse og evne til at detektere ikke-levedygtige bakterier øge risikoen for positive testresultater uden klinisk relevans. Derfor bør BU vælges, hvis en høj Sp ønskes. Pattehuden som reservoir skal overvejes, særligt ved tolkning af testresultater fra ikke-sterilt indsamlede mælkeprøver, og SCC bør bruges som supplement til BU eller PCR ved diagnosticering af IMI. Generelt bør diagnoser stilles på baggrund af gentagne prøver for at kunne tage højde for det tidsmæssige perspektiv af infektionen og for at minimere risikoen for at stille forkerte diagnoser.

List of abbreviations and terms

AMS	automatic milking system
BC	bacterial culture
BTM	bulk tank milk
Cfu	colony forming units
Ct	cycle threshold
DIM	days in milk
DTU-VET	Technical University of Denmark (National Veterinary Institute)
LCA	latent class analysis
MALDI-TOF	Matrix-assisted laser desorption-ionisation time of flight
MLST	multilocus sequence typing
PCR	polymerase chain reaction
RQ	research question
Se	sensitivity
SCC	somatic cell count
Sp	specificity
ST	sequence type

1 Introduction

Mastitis is the most common disease in dairy herds, leading to high economic losses due to reduced milk production and quality, increased treatment costs, and involuntary culling (Halasa et al., 2007; Seegers et al., 2003). Furthermore, mastitis causes a decrease in dairy cow welfare (EFSA, 2009), and approximately 70% of the antibiotics used in dairy cattle in Denmark are prescribed for mastitis (DANMAP, 2016). Therefore, effective mastitis control is a cornerstone in improving animal welfare, ensuring milk quality, and reducing antibiotic usage.

With rapidly increasing herd sizes, focus on contagious udder pathogens becomes important. *Streptococcus agalactiae* and *Staphylococcus aureus* are the main udder pathogens considered to be contagious (Keefe, 2012). Even though many herds have successfully controlled *Strep. agalactiae* and *Staph. aureus* through implementation of standard mastitis prevention programmes (Barkema et al., 2009), problems with contagious mastitis continue in some parts of the world.

Based on an annual test of bulk tank milk (BTM) in Danish dairy herds, an increase was observed in the apparent prevalence of herds infected with *Strep. agalactiae* from 2% in 2000 to 7% in 2010, and approximately 90% of Danish herds were positive for *Staph. aureus* in 2010 (Katholm et al., 2012). The re-emergence of *Strep. agalactiae* is of particular concern, and explanations could include the rapid increase in the size of Danish herds and the increase in the proportion of herds using automatic milking systems (AMS), which have a higher risk of being positive for *Strep. agalactiae* compared to herds with conventional milking systems (Katholm, 2010). Furthermore, a recent study demonstrated that *Strep. agalactiae* is present in the barn environment (Jørgensen et al., 2016), which had not previously been considered a reservoir. This could be related to a shift in sequence types (ST) of the pathogens affecting dairy cows. Mainly human strains of *Strep. agalactiae* were recently isolated from dairy cattle (Zadoks et al., 2011), and these strains may have other pathogenic characteristics than the previously isolated bovine strains.

The most effective control of contagious udder pathogens with a high risk of spread within a herd and resulting in a large number of infected quarters relies on early and valid diagnosis (Barkema et al., 2009). Efficient, feasible and cost-effective diagnostic tools and management practices are of special importance in large herds to ensure early detection of pathogens and prompt implementation of control strategies so that the spread of pathogens, discarding of milk and high labour costs can be avoided.

Early and valid diagnosis of mastitis or intramammary infections (IMI), however, depends on several factors. The purpose of the diagnosis may influence the need for high sensitivity (Se; e.g. in eradication programmes) or high specificity (e.g. if culling is a consequence of a positive test). It is important to have an appropriate sampling frame on which to base the diagnosis, as well as good test performance. Detection of contagious udder pathogens may be challenged by the tendency of the bacteria to be excreted from the mammary gland in a cyclic pattern. This has been demonstrated for *Staph. aureus* (Sears et al., 1990; Studer et al., 2008) and *Strep. agalactiae* (Thieme and Haasmann, 1978), which is why repeated sampling has been recommended in order to increase the Se for detection of *Staph. aureus* IMI (Buelow et al., 1996; NMC, 2004; Sears et al.,

1990). However, guidelines for sampling and the interpretation of test results, as well as the precise definition of IMI are still lacking (Andersen et al., 2010; NMC, 2004).

Furthermore, there are various test systems and methods available and these can all be interpreted differently. A general problem relating to the evaluation of test performance for IMI detection is the lack of a reference standard. Bacterial culture (BC) is the most frequently used diagnostic tool for identification of udder pathogens (Lam et al., 2009). However, the polymerase chain reaction (PCR) test has been used more frequently in recent years – especially in the Nordic countries (Koskinen et al., 2010; Mahmmod et al., 2013b, 2013c; Nyman et al., 2016). The PCR test is a tool that can rapidly detect even small numbers of bacterial DNA from a number of different pathogens in milk, it is user independent, and it can facilitate automation in the laboratory workflow (Gillespie and Oliver, 2005; Koskinen et al., 2009), meaning that the cost in Denmark is comparable with the cost of BC. In contrast, BC is time-consuming and can easily require up to 48 hours to identify pathogens (NMC, 2004). Furthermore, an annual voluntary external quality assurance of BC conducted among Danish herd veterinarians' laboratories has shown that accurate identification of udder pathogens could be improved. For example the proportion of attending veterinarians that correctly identified *Strep. agalactiae* was as low as 54 % in 2006 (Karlsmose et al., 2013).

Since 2009, Danish farmers have been able to order cow-level PCR testing (PathoProof™) as part of the routine milk recording scheme (Katholm, 2010), and evaluation of the PCR test with BC has shown that PCR has high analytical Se (Koskinen et al., 2010; Mahmmod et al., 2013b, 2013c). However, milk samples ordered through the routine milk recording scheme are not aseptically collected, and there is a risk of carryover and contamination (Mahmmod et al., 2017). As the PCR test detects only bacterial DNA, there is a risk that non-viable bacteria with no clinical importance will also be detected (Koskinen et al., 2009).

A solid understanding of the sources and transmission of the organisms is also crucial for an effective control programme. The role of the teat skin – both as a reservoir of pathogens and in transmission – must be known if effective measures are to be taken to break transmission. Furthermore, the relationship between pathogens present on teat skin and in milk is central to validating the PCR diagnostic used in routine milk testing. If there are pathogens on the teat skin but not in the milk, then there is an increased risk of a false-positive test result when the milk samples are not aseptically collected. The association between *Strep. agalactiae* on teat skin and in milk has not previously been investigated. An association has been reported for *Staph. aureus*, but this was based only on BC (da Costa et al., 2014; Haveri et al., 2008; Piccinini et al., 2009). To examine the relationship between both *Strep. agalactiae* and *Staph. aureus* on teat skin and in milk, the PCR test must be validated using non-milk samples.

More knowledge on pathogen dynamics, pathogen reservoirs and test performance is required given the pathogen dynamics and potential existence of reservoirs under field conditions. With this, we can begin to improve the diagnosis and control of *Strep. agalactiae* and *Staph. aureus* in Danish dairy herds, develop guidelines for sampling, choose a suitable diagnostic test and provide an appropriate interpretation of the results in relation to a given purpose.

1.1 Aim and objectives

The overall aim of this PhD project was to gain more knowledge about the dynamics of the contagious udder pathogens *Strep. agalactiae* and *Staph. aureus* in order to improve recommendations for the efficient diagnosis and control of contagious mastitis in dairy herds.

The overall aim was addressed through several sub-studies with the following research questions (RQ):

RQ1: What is the association between teat skin colonisation and intramammary infection with *Staph. aureus?*

RQ2: What is the association between teat skin colonisation and intramammary infection with *Strep. agalactiae*?

RQ3: What is the diagnostic accuracy of PCR and BC for identifying *Staph. aureus* in milk and teat skin samples?

RQ4: What is the diagnostic accuracy of PCR and BC for identifying *Strep. agalactiae* in milk and teat skin samples?

RQ5: What is the short-term diagnostic test pattern of naturally occurring intramammary infections with *Staph. aureus* and *Strep. agalactiae* ?

RQ6: Which infection types do mastitis experts identify based on different diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae*?

RQ7: How well do PCR, BC and somatic cell count (SCC) tests perform in identifying the different infection types established by mastitis experts?

1.2 Outline of the thesis

This thesis is based on three manuscripts and has the following structure:

Chapter 2 presents current knowledge about *Staph. aureus*, *Strep. agalactiae* and the diagnostic methods related to detecting these pathogens in milk.

Chapter 3 gives an overview of the sampling activities and the materials and methods used in the different studies. See also Figure 1.1.

Chapter 4 presents the main findings of the studies.

Chapter 5 is a general discussion and includes recommendations and perspectives.

Conclusions are presented in **Chapter 6**.

References can be found in **Chapter 7**.

Chapter 8 contains the three manuscripts:

Manuscript I: Association between teat skin colonization and intramammary infection with *Staphylococcus aureus* and *Streptococcus agalactiae* in herds with automatic milking systems

Manuscript II: Accuracy of qPCR and bacterial culture for the diagnosis of bovine intramammary infections and teat skin colonisation with *Streptococcus agalactiae* and *Staphylococcus aureus* using Bayesian analysis

Manuscript III: Expert evaluation of different mastitis profiles in dairy cow quarters naturally infected with *Staphylococcus aureus* or *Streptococcus agalactiae*

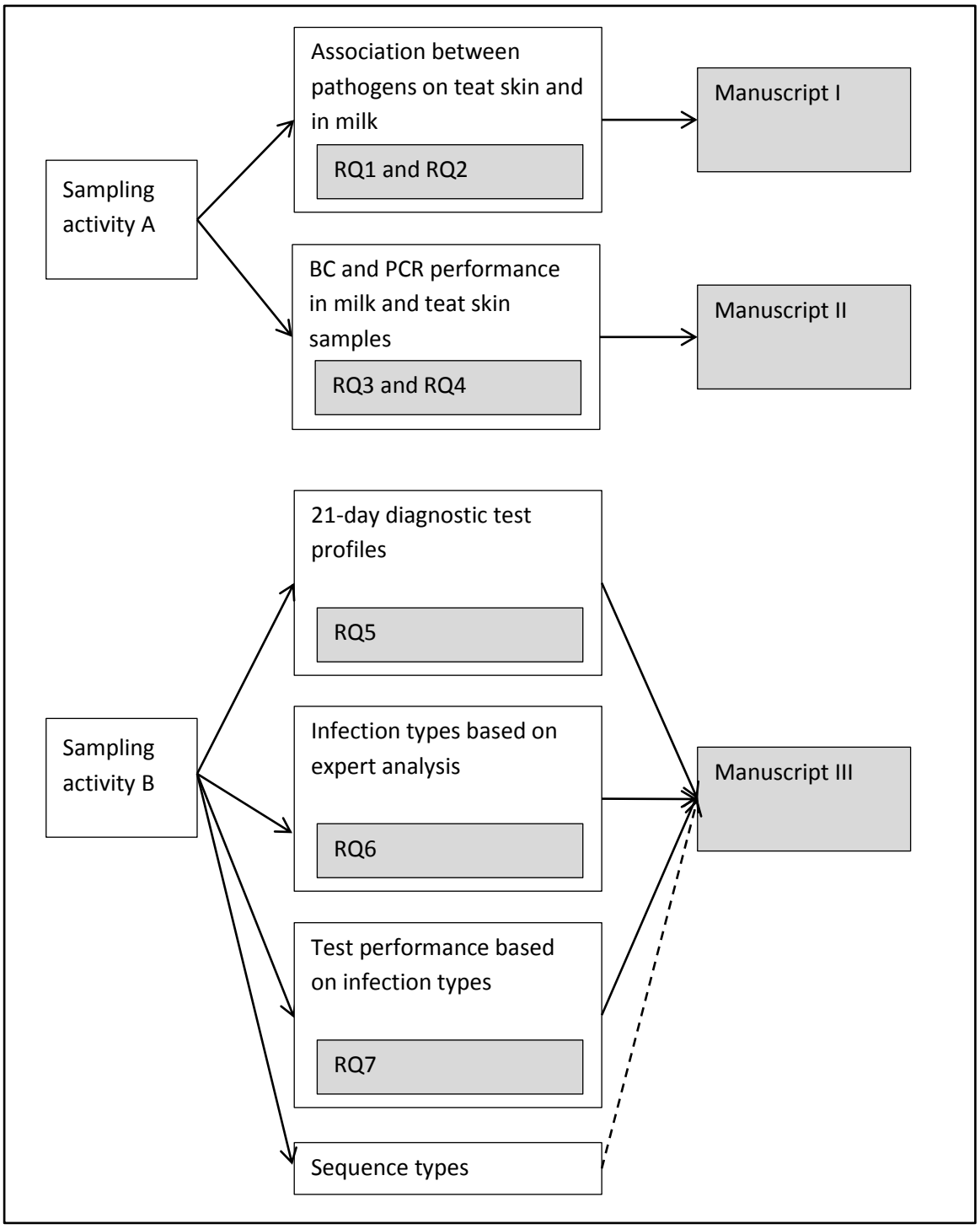


Figure 1.1: Overview of study including research questions (RQ), sampling activities and manuscripts

2 Background

This section provides a brief overview of mastitis with *Staph. aureus* and *Strep. agalactiae,* with a focus on diagnosis and diagnostics.

2.1 Mastitis and intramammary infections

Mastitis and IMI are terms that are used interchangeably (Andersen et al., 2010). However, the definitions provided by the International Dairy Federation (IDF) define the difference: IMI is categorised as an infection occurring in the mammary gland, whereas mastitis defines inflammation in the mammary gland, almost always caused by infectious microorganisms. IMI thereby covers the presence of pathogens in the udder, and it is recommended that these pathogens should be diagnosed by BC of aseptically obtained milk samples (IDF, 2011). In contrast, mastitis can be present even when it is not possible to isolate an udder pathogen from the milk. Clinical mastitis is characterised by clots in the milk or other signs of inflammation in the udder like heat, swelling and pain (Harmon, 1994). Therefore, clinical mastitis is diagnosed through visual observation or physical examination of the milk and udder, whereas detection of subclinical mastitis depends on a measure of inflammation indicators in milk e.g. via SCC or California Mastitis Test (IDF, 2011).

Udder pathogens are traditionally divided into a contagious and an environmental group according to their main route of transmission (during milking or between milkings). *Staph. aureus* and *Strep. agalactiae* are considered contagious udder pathogens, whereas all others are considered environmental (Harmon, 1994; Smith et al., 1985).

Udder pathogens enter the mammary gland through the teat canal. Under normal conditions, a small number of somatic cells are present in the udder and they attempt to combat the pathogens immediately. Bacteria and leukocytes of the infected quarter will release chemo attractants for leukocytes, and neutrophils from the bloodstream will move rapidly to the udder and cause an immediate increase in SCC. In many cases, this will eliminate the pathogens and only a mild inflammatory episode (mastitis) will be required to restore a healthy gland. In some cases, however, the immune system is insufficient: bacteria will multiply and an IMI will manifest (Harmon, 1994; Rainard and Riollet, 2006). The mean SCC of a healthy gland has been reported at approximately 70,000 cells/mL (Schukken et al., 2003), however, the level highly depends on milk production, lactational stage and parity (Græsbøll et al., 2016; Schukken et al., 2003). The cut-off 200,000 cells/mL is the mainly used for practical purposes to distiguish healthy from inflammatory quarters (Schukken et al., 2003).

The teat canal acts as a barrier against the entrance of pathogens to the mammary gland. Together with the sphincter muscles at the teat end, a keratin plug works as a physical barrier, preventing the penetration of bacteria. At milking, the teat end sphincter opens and requires time after milking to contract (Rainard and Riollet, 2006). Poor teat end condition (e.g. hyperkeratosis, teat congestion and oedema induced by the milking process) increases the period for which the teat ends are open after milking, thereby increasing the risk of IMI (Mein et al., 2014). Furthermore, teat end hyperkeratosis can be associated with the bacterial load at the teat end (Paduch et al., 2012).

Bacteria present on teat skin and udder pathogens colonising the teat canal may cause a positive milk sample even though an IMI is not present (Hiitiö et al., 2016). Studies have found teat skin and teat canal colonisation with several pathogens including *Staph. aureus* (Haveri et al., 2008; Paduch et al., 2012), and it has been demonstrated that milk samples collected directly from the udder cistern have a lower number of different microorganisms compared to samples aseptically collected through the teat canal (Hiitiö et al., 2016). A positive milk sample may therefore represent "contamination" with bacteria from the teat canal and teat skin, rather than an IMI. Furthermore, variable shedding of bacteria in the milk has been described for *Staph. aureus* (Sears et al., 1990; Studer et al., 2008) and *Strep. agalactiae* (Thieme and Haasmann, 1978), which means that an IMI can be present without pathogens being detected in the milk. For *Staph. aureus,* this phenomenon is explained by the ability of the bacteria to survive and hide intracellularly (Godden et al., 2002; Studer et al., 2008; Zecconi et al., 1997), which means that a cow or quarter can "carry" an infectious agent without shedding it.

2.1.1 Staphylococcus aureus

Staph. aureus is one of the most common causes of mastitis in cattle globally. The estimated herd-level prevalence in Denmark, Germany, Belgium and Canada has been reported at 91% (Katholm et al., 2012), 90% (Tenhagen et al., 2006), 86% (Piepers et al., 2007) and 74% (Olde Riekerink et al., 2006), respectively. Within-herd prevalence has been reported to range from 0 to 40.3% (Mahmmod et al., 2013a; Piepers et al., 2007). Quarter-level prevalence has been reported at 3.1% (Piepers et al., 2007), 5.7% (Tenhagen et al., 2006) and 8.2% (Osterås et al., 2006), in all cases with *Staph. aureus* as the most or second most frequently isolated pathogen at quarter level. In milk samples from cows with high SCC (≥ 250,000 cells/mL), quarter-level *Staph. aureus* IMI was reported at 4.2% (Sampimon et al., 2009) and 7.6% (Piepers et al., 2007). The prevalence of *Staph. aureus* in clinical milk samples has been reported at 3.3% (Bradley et al., 2007), and 10.3% (Olde Riekerink et al., 2008). Timonen et al. (2017) reported a within-herd prevalence assessed by PCR of 10.2% in Estonian herds, whereas Mahmmod et al. (2013a) reported a within-herd prevalence of between 16% and 48% in six Danish dairy herds assessed by PCR.

Staph. aureus is a commensal and opportunistic pathogen that infects humans and animal species (Rainard et al., 2017). In dairy cows, the main disease caused by *Staph. aureus* is mastitis, but it can also cause a variety of other diseases (Rainard et al., 2017; Zadoks et al., 2011). The bacterium is a facultative anaerobic Gram-positive coccus that can grow on calf blood agar under aerobic conditions. *Staph. aureus* is catalase and coagulase positive, and colonies are identified as 3 to 5 mm large, creamy, golden-yellow or greyish-white on blood agar. *Staph. aureus* is mainly beta-haemolytic, but may be non-haemolytic or present alfa-haemolysis as a narrow zone of complete haemolysis around the colonies, and/or beta-haemolysis as a wider zone of incomplete haemolysis (NMC, 2004).

Staph. aureus causes problems due to its pathogenicity, contagiousness, persistence in the bovine environment, colonisation of skin and poor cure rates (Rainard et al., 2017). In dairy cows, *Staph. aureus* mastitis is mainly subclinical with moderately elevated SCC, although clinical mastitis can occur, including severe and gangrenous cases with lethal outcomes (Keefe, 2012). Furthermore,

Staph. aureus mastitis is often chronic and the cure rate for antibiotic treatment is reported to be as low as 3% depending on several cow- and pathogen-specific factors (Barkema et al., 2006).

Chronically infected mammary glands are the main reservoir of *Staph. aureus* within herds, and it is thought that transmission mainly occurs through the milking equipment that is contaminated with *Staph. aureus* from the milk, teat skin or teat canal of the previously milked cows (Haveri et al., 2008; Zadoks et al., 2002). However, *Staph. aureus* has also been isolated from the skin and skin lesions of cows and heifers, the barn environment (including bedding, water and milking equipment), flies and from milkers' hands and nostrils (Fox et al., 1991; Larsen et al., 2000; Roberson et al., 1994). The role of reservoirs outside the mammary gland has been discussed, as strains found in milk and on teat skin have been reported as both the same (Haveri et al., 2008; Piccinini et al., 2009) and different (Zadoks et al., 2002). However, molecular studies suggest the potential for both contagious and environmental transmission for *Staph. aureus*, as several different strains have been isolated from the same herds and cows, which is more in line with the behaviour of environmental than contagious pathogens (Zadoks et al., 2011).

2.1.2 Streptococcus agalactiae

Eradication is often mentioned as a goal in the control of *Strep. agalactiae* mastitis (Keefe, 2012). Despite successful efforts to reduce the prevalence of *Strep. agalactiae* in Scandinavian countries during the 20th century, the prevalence of *Strep. agalactiae*-positive herds in Denmark and Norway has increased during the early 21st century (Katholm et al., 2012; Mweu et al., 2012; Radtke et al., 2012). The estimated herd-level prevalence of *Strep. agalactiae* has been reported at 7% (Katholm et al., 2012), 29% (Tenhagen et al., 2006), 5.3% (Piepers et al., 2007), and 1.6% (Olde Riekerink et al., 2006) in Denmark, Germany, Belgium and Canada, respectively. In a study of six Danish dairy herds, the cow- and quarter-level prevalence was reported at 7.8% (ranging from 1.6 to 21.7%) and 2.8% (ranging from 0.4 to 7.8%), respectively (Mahmmod et al., 2015). In Germany, a cow-level prevalence of 0.7% was reported (Tenhagen et al., 2006) and in Belgium, a prevalence of 0.1% was reported at both cow and quarter level, whereas *Strep. agalactiae* was not isolated from 480 clinical mastitis milk samples from British herds (Bradley et al., 2007), nor from 2,174 milk samples from subclinical mastitis in Dutch herds (Sampimon et al., 2009). Using PCR, Timonen et al. (2017) reported a cow-level prevalence of 28.4% in Estonian herds, and Mahmmod et al. (2015) reported a cow-level prevalence of 27.4% in Danish herds.

Strep. agalactiae is a facultative anaerobic Gram-positive coccus that can grow on calf blood agar under aerobic conditions. It is catalase negative and produces smooth, translucent, greyish-white colonies of 1 to 3 mm in size. *Strep agalactiae* can be non-haemolytic or with alfa-haemolysis, but most produce beta-haemolysis (Daignault et al., 2003). *Strep. agalactiae* has the ability to produce complete haemolysis of calf blood erythrocytes in the presence of *Staph. aureus* toxin, which is the principle used in the CAMP (Christie – Atkins – Munch-Petersen) test recommended for identification of *Strep. agalactiae* (Keefe, 1997).

Strep. agalactiae IMI present mainly as a subclinical mastitis with high SCC, and the amount of bacteria shed from an infected udder quarter is expected to be high (Keefe, 2012). *Strep. agalactiae* is considered a contagious udder pathogen related to the bovine mammary gland, with

transmission among cows occurring during milking (Keefe, 1997). However, *Strep. agalactiae* has been recovered from the skin of several different body parts of the cow, from milkers' hands and clothes and from the barn environment (Chodkowski, 1949). Furthermore, recent studies have shown the same possible reservoir of *Strep. agalactiae* in the barn environment, e.g. stalls and water troughs and extramammary body parts like the rectum and vagina (Farre et al., 2017; Jørgensen et al., 2016). In addition, different components of milking equipment (in AMS) presented *Strep. agalactiae* (Henriksen et al., 2017; Jørgensen et al., 2016).

Strep. agalactiae colonises the urogenital and gastro-intestinal tract of humans (asymptomatic) and is mainly associated with disease (septicaemia) in infants (Keefe, 1997). *Strep. agalactiae* has also been associated with other domestic animals, e.g. cats and dogs (Keefe, 2012; Zadoks et al., 2011), so humans and animals other than cows may act as a source of the bacteria in dairy cattle herds. Using molecular methods, the same type of *Strep. agalactiae* was found within herds, confirming the contagious pattern of transmission (Jørgensen et al., 2016; Mahmmod et al., 2015; Zadoks et al., 2011). However, it is still debated whether cows and humans share a reservoir of *Strep. agalactiae*, and whether the strain type within a herd may be associated with epidemiological and pathogenic behaviour (Lyhs et al., 2016; Mahmmod et al., 2015; Zadoks et al., 2011).

2.2 Prevention and control of *Staphylococcus aureus* and *Streptococcus agalactiae* mastitis

As *Staph. aureus* and *Strep. agalactiae* are considered contagious pathogens spreading from infected to healthy udder quarters, they should be effectively controlled by implementing the "five-point-plan", which has been further developed to include ten points (National Mastitis Council, n.d.). The control plan is built on two principles, described by Neave et al. (1966): 1) reducing new cases of infection during lactation using simple hygiene approaches, and 2) elimination of established infections and new infections through treatment. Both the five- and ten-point plans include: proper cleaning of teats including post-milking teat disinfection, proper use and maintenance of milking machines, treatment of mastitis including dry cow treatment, and culling of chronically infected cows (Biggs, 2009; National Mastitis Council, n.d.). Furthermore, within- and between-herd biosecurity (including identification and segregation of infected cows as well as avoiding introduction of infected cows to the herd) is important (Barkema et al., 2006; Keefe, 2012) as it has been shown that prevalence and incidence rates are associated (Dufour et al., 2012).

Lactational treatment and dry cow therapy are included in the control programmes of both *Staph. aureus* and *Strep. agalactiae*, but due to the low cure rate, lactational treatment should be used for *Staph. aureus* only after taking into account several different cow- and herd-specific factors (Barkema et al., 2006; Keefe, 2012). In Denmark, dry cow therapy and lactational treatment with antibiotics other than penicillin require milk samples with detected pathogens (BEK nr 1353 af 29/11/2017, n.d.; BEK nr 1537 af 12/12/2016, n.d.), and as such, treatment in Denmark should rely on a positive milk sample.

In Denmark, a surveillance program based on BTM testing for *Strep. agalactiae* has run since 1954. It was introduced along with an eradication program including; identification of infected cows by quarter milk samples, treatment and culling of infected cows, and improvement of milking procedures and hygiene (Andersen et al., 2003). The eradication program was compulsory until 1988 and has since been voluntary, and a positive *Strep. agalactiae* status has now minimal prohibitions regarding disclosure (BEK nr 225 af 17/03/2005, n.d.).

2.3 Diagnostic tests

IMI can be detected in different ways, and the following section describes the common practice in Denmark. To detect pathogens causing clinical mastitis, BC is typically carried out by the herd veterinarian based on an aseptically collected milk sample (collected by the veterinarian or the farmer) from a quarter with signs of clinical mastitis. In veterinary practice, the identification of pathogens is mainly based on colony morphology on blood agar and/or a chromogenic agar. In addition, sensitivity to penicillin is often tested using penicillin agar plates for culture. Voluntary external quality assurance has revealed that inaccurate diagnosis of *Staph. aureus* and *Strep. agalactiae* appears to be common. Furthermore, the types of incorrect diagnoses indicate that basic biochemical tests are not routinely used (Karlsmose et al., 2013).

Since 2009, it has been possible to order routine PCR tests at a commercial laboratory (Eurofins Steins, Vejen, Denmark) that determine e.g. SCC analysis included in the milk recording. This is mainly used for testing non-aseptically collected composite milk samples from the milk recording of cows nearing dry-off. The PCR test result is then used to select cows for dry-off treatment. In 2016, 70,000 cow-level milk samples from milk recordings were tested using PCR for dry-off purposes (RYK, 2016).

SCC is measured during milk recording, for which 90% of Danish milk-producing farms are voluntarily included for cow-level sampling either 6 or 11 times per year (RYK, 2016). In relation to mastitis diagnostics, the SCC test results are primarily used for monitoring subclinical mastitis, and in some cases to select cows for dry cow therapy. Milk recording is performed on non-aseptically, automatically collected composite milk samples.

2.3.1 Bacterial culture (BC)

The principle of BC is detection of viable microbial cells. To detect udder pathogens, an aliquot of milk is spread on an agar plate and incubated at 37°C for typically 24 to 48 h. However, some organisms need a longer incubation, anaerobic conditions or special nutrition for growth (NMC, 2004). The identification of colonies is based on morphology and biochemical tests. If growth of more than two different species is observed, the milk sample or agar plate should be considered contaminated, even though co-infections are a possibility (NMC, 2004). A disadvantage of BC is that it is time-consuming. Furthermore, the quality depends on the “laboratory” (Karlsmose et al., 2013).

In addition to BC, confirmatory tests like antigen-antibody agglutination or Matrix-assisted laser desorption-ionisation time of flight (MALDI-TOF) could be applied in order to identify udder pathogens at species level (Mahmmod et al., 2018; Nonnemann et al., 2018).

BC is considered the reference method for the detection of udder pathogens. The fact that BC detects viable bacteria in milk is in agreement with the IMI definition (IDF, 2011). However, a potential problem with BC is that no growth of bacteria is obtained in clinical or subclinical mastitis samples (Bradley et al., 2007). This could be due to growth-inhibitory compounds of the milk and immune response (Rainard and Riollet, 2006), or due to disinfectant residue or antimicrobials from treatment. Furthermore, in relation to assessing IMI, the interpretation of BC results is not concise (Andersen et al., 2010). For example, the method used for BC, the concentration of bacteria present in the milk sample and the number of samples necessary to confirm an IMI can vary. The choice of method should depend on the reason the sample was collected and the related aims (Andersen et al., 2010; Dohoo et al., 2011). For detection of IMI with *Staph. aureus,* repeated samples are recommended in order to increase the chance of detecting IMI (Buelow et al., 1996; Nyman et al., 2016). This is mainly due to variation in the shedding of bacteria (Sears et al., 1990; Studer et al., 2008). Furthermore, freezing, increased inoculated volume and centrifugation of milk samples have shown potential to increase the chance of growing *Staph. aureus* (Godden et al., 2002; Walker et al., 2010; Zecconi et al., 1997). A concentration of 1,000 cfu/mL has been suggested as the detection limit of IMI (Andersen et al., 2010), whereas for pathogens other than coagulase-negative Staphylococci (CNS), 100 cfu/mL should be enough to define IMI according to Dohoo et al. (2011). Following the most commonly used protocol for BC (NMC, 2004), 0.01 mL of milk should be plated and one colony detected – corresponding to 100 cfu/mL being considered a positive sample.

When BC is compared to PCR, the Sp estimates are generally reported to be high, whereas Se estimates are low. Test performance estimates from a number of studies on *Staph. aureus* and *Strep. agalactiae* are shown in Table 2.1.

2.3.2 Polymerase Chain Reaction (PCR)

In contrast to BC, PCR methods focus on nucleic acid composition of the bacterial genome (DNA), rather than the phenotypic expression of what the DNA encode. Therefore, PCR should be more accurate than BC (Gillespie and Oliver, 2005).

The principle of PCR is to produce multiple copies of a target sequence of DNA through multiple cycles of transcription. The PCR cycle consists of three steps at different temperatures. In the first step (denaturation), the coiled DNA strands are separated. In the second step (annealing), the primers should bind to the opposite template DNA strands. In the third step (extension), the new DNA strand is synthesised by the polymerase enzyme. Under optimal conditions, the number of target DNA will be doubled during each cycle. The DNA must be identified during or after the amplification process. This can be done quantitatively in a real-time PCR test, in which primers with fluorescence are used. The fluorescent signal produced from the sample is plotted against the PCR cycle number and only samples above a certain fluorescence threshold are defined as positive. The Ct value then represents the number of PCR cycles required to reach this particular threshold. The fewer PCR cycles required to reach the threshold, the more bacterial DNA will be present in the sample (Tang and Stratton, 2013).

Several primers can be added to the test to detect several targets or species (multiplex real-time PCR) (Gillespie and Oliver, 2005). The specificity (Sp) of PCR should be high if primers are properly designed for the target and misidentification is unlikely. The Se of PCR should be low compared to BC, as a smaller volume of the original sample is analysed, yet problems with overgrowth (contamination) and special growth requirements can be avoided (Britten, 2012). Furthermore, in addition to viable bacteria, the PCR assay can also detect growth-inhibited and non-viable bacteria, which is also likely to increase the Se compared to BC (Koskinen et al., 2009; Taponen et al., 2009). Whether this detection is relevant from a clinical point of view is debatable, as it might be dead bacterial DNA that is present days or weeks after an infection has resolved (Britten, 2012; Hiitiö et al., 2018).

The PCR assay has been evaluated for the detection of udder pathogens including *Staph. aureus* and *Strep. agalactiae*. Methods differ among studies, with automatically (non-aseptically) or aseptically collected cow- or quarter-level milk samples and Latent Class Analysis (LCA) or BC used as gold standard (Table 2.1). The Se of PCR has generally been shown to be high compared with BC. Sp estimates of PCR should, however, be interpreted with caution when non-aseptically collected milk samples are used, as carry-over and contamination may occur (Mahmmod et al., 2017, 2014).

Table 2.1: References from the literature reporting sensitivity (Se) and specificity (Sp) estimates for polymerase chain reaction (PCR) and bacterial culture (BC) detecting *Staphylococcus aureus* and *Streptococcus agalactiae* in milk samples from naturally infected dairy cows

Reference	Pathogen	Se_{PCR}[a]	Sp_{PCR}[a]	Se_{BC}	Sp_{BC}
(Cederlöf et al., 2012) - BC aseptically collected - PCR automatically collected - Evaluated at cow level in LCA - Cows at dry-off	*Staphylococcus aureus*	93.0	95.0	83.0	97.0
(Mahmmod et al., 2013c) - BC aseptically collected - PCR automatically collected - Evaluated at cow level in LCA	*Staphylococcus aureus*	90.8	89.5	52.3	98.8
(Nyman et al., 2016) - BC aseptically collected - PCR automatically collected - Evaluated at cow level in LCA[b]	*Staphylococcus aureus*	86 (80)[c]	98 (97)[c]	64 (83)[c]	98 (95)[c]
(Steele et al., 2017) - BC and PCR aseptically collected - Evaluated at quarter level - BC as gold standard	*Staphylococcus aureus*	96.4	99.7	NA	NA
(Mahmmod et al., 2013b) - BC aseptically collected - PCR automatically collected - Evaluated at cow level in LCA	*Streptococcus agalactiae*	91.9	96.9	29.9	99.5
(Holmøy et al., 2018) - BC and PCR aseptically collected - Evaluated at cow level in LCA[b]	*Streptococcus agalactiae*	93.3	98.5	39.0	99.6

[a] Cut-off Ct value ≤ 37; [b] results from LCA using informative priors; [c] BC considered positive based on at least one out of three consecutive milk samples

3 Materials and Methods

The data were collected during two sampling activities (Figure 1.1). This chapter provides an overview of these activities, which are described in detail in the respective papers.

3.1 Sampling activity A

This sampling aimed to support the investigation of teat skin as a reservoir of *Staph. aureus* and *Strep. agalactiae,* using both PCR and BC as diagnostic tests. In addition, the two tests should be validated for both milk and teat skin samples. The sampling process is also described in Manuscripts I and II.

3.1.1 Herds and animals

The sample included AMS herds, as the association between teat skin colonisation and IMI with *Staph. aureus* was previously only investigated in herds with conventional milking systems. Furthermore, unknown circumstances appear to increase the risk of AMS herds being *Strep. agalactiae* positive compared to conventional milking (Katholm, 2010).

We aimed to sample cows with a high probability of being infected with *Strep. agalactiae*, which was expected to be less prevalent than *Staph. aureus*. However, we were also interested in quarters with no infection present to determine whether the teat skin can be colonised even if no pathogens are present in the milk. To achieve a sample of cows with different infection statuses but with a high risk of infection, we decided to sample from cows with high SCC, i.e. a random sample of cows with SCC $\geq$ 200,000 cells/mL at the latest milk recording.

The sample size was calculated in a cross-sectional set-up. With the aim to identify a relative risk of 2, and under the assumption that the prevalence of high-SCC cows with *Strep. agalactiae* IMI was 10% among cows without teat skin colonisation, a sample size of 314 cows would be required.

The selection criteria for the study herds were: 1) AMS herds with Cycle threshold (Ct) < 32 for *Strep. agalactiae* in the last annual (2016) BTM test for *Strep. agalactiae* surveillance, 2) at least three AMS units or 150 cow-years and 3) willingness from the farmer to participate. Based on a list of Danish AMS herds with positive *Strep. agalactiae* status extracted from the Danish Cattle Database, 28 farms met the criteria. Five farms were excluded; one was located on Bornholm, two farms were declared bankrupt and two farms were already participating in another STOPMAST herd trial. The remaining 23 farms were contacted by letter, and eight responded positively. Before inclusion in the study, BTM from the eight herds was tested with PCR (Mastit4, DNA Diagnostic) to make sure they were still positive for *Strep. agalactiae*. The herds had to be positive (i.e. with a Ct < 32) in at least two out of three weekly tests. All eight herds were positive and therefore included in the study.

3.1.2 Teat skin and milk samples

The participating herds were located throughout Jutland. The laboratory facilities were at the Danish Veterinary Institute, Technical University of Denmark (DTU-VET), Copenhagen (Zealand), and the samples for PCR analysis could be shipped from here to DNA Diagnostic (Aarhus, Jutland, Denmark). The sampling and laboratory analyses were carried out from February to May 2017.

With the exception of a break over the Easter period, a herd was visited every week with the following schedule:

- Day 1) Herd visit (sampling)
- Day 2) Plating of samples in the DTU-VET laboratory and shipment of samples for PCR testing at DNA Diagnostic (performed 1 or 2 days later)
- Day 3) First reading of bacteriology plates
- Day 4) Second and final reading of plates, with final bacterial identification
- Day 5) Harvesting of isolates (with subsequent storage in glycerol) for future typing

Sampling

Before each herd visit, a list of 30 to 40 cows randomly selected from all cows with SCC > 200,000 cells/mL at the most recent milk recording were created. Cows treated with antibiotics within the last 4 weeks were not included. The farmers were asked to separate (for sampling) as many cows from that list as possible, but they should not include dry cows or cows that had been treated with antibiotics since the last milk recording.

To remove dirt from the teat skin without removing the bacteria colonising it, the teats were cleaned with dry paper towels (udder paper) before sampling. The teat skin samples were collected using the modified wet-and-dry method described by Paduch and Kroemker (2011). Both a wet and a dry rayon swab (DaklaPack, Glostrup, Denmark) were rotated 360° around the teat approximately 1 cm from the teat canal orifice. Both swabs were broken off into a tube containing 2 mL of ¼ Ringer's solution (Merck, Darmstadt, Germany). All milking quarters of each selected cow were sampled. Following the collection of teat skin samples, but before quarter-level milk samples were aseptically collected, the occurrence of hyperkeratosis was scored using a 4-point scale (Mein et al., 2001).

Gloves were changed between each cow and sampling procedure. The teat skin samples were collected by me and two trained veterinary master's students, who also scored the teat ends for hyperkeratosis. Each of the individuals involved in the teat skin sampling worked in a team with a milk quality technician, who collected the milk samples. The samples were cooled in ice boxes and stored at 5°C until arrival at the laboratory the following day.

Laboratory procedures

Both milk and teat skin samples were cultured on three different media: 1) blood agar (5% sheep blood), 2) chromogenic agar selective for staphylococci (SaSelect, Bio-Rad, Hercules, CA), and 3) modified Edwards medium [Oxoid, Roskilde, Denmark, supplemented with 5% calf blood and 2% filtrate of a β-toxin producing *Staph. aureus* prepared as described by Jørgensen et al. (2016)]. For each medium, 0.01 mL of milk was streaked on a quarter of a plate, and 0.1 mL of the teat skin sample was pipetted and spread on a whole plate. BC was carried out by me, the two veterinary master's students and a postdoc.

Immediately after culturing, a subsample of the milk and teat skin samples from right rear quarters were taken for PCR analysis. This was done by immersing a FLOQswab (COPAN ITALIA spa, Brescia, Italy) into each sample. The swabs were able to soak up 0.22 mL of fluid and would

dry out quickly, which meant they could be shipped without cooling. The PCR analysis was carried out by DNA Diagnostic (Aarhus, Denmark) using the Mastit4 test kit (named Mastit4BDF) containing primers for *Staph. aureus, Strep. agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Mycoplasma bovis, Mycoplasma* spp., coagulase negative staphylococci (CNS), β-lactamase gene, *Prototheca*, *Escherichia coli*, *Enterococcus* spp., and *Lactococcus lactis* subsp. *lactis*, and *Klebsiella* spp. (*pneumoniae, oxytoca* and *variicola*).

The plates for BC were read at 24 and 48 h of incubation. *Strep. agalactiae* colonies were phenotypically identified on blood agar as small (1 to 3 mm), greyish-white and with no haemolysis, alfa or beta haemolysis, or on the modified Edwards medium as aesculin-negative, blue-to-colourless colonies with a CAMP (Christie – Atkins – Munch-Petersen) reaction. Identification of *Strep. agalactiae* was confirmed with either a slide agglutination test for Lancefield group B (PathoDxtra Strep Grouping Kit, ThermoFisher Scientific, Waltham, MA) or MALDI-TOF (Bruker Biotyper software system, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany). *Staph. aureus* was phenotypically identified on blood agar as large (3 to 5 mm), greyish-white or yellow and with different degrees of haemolysis, or on SaSelect agar as pink colonies. Identification was confirmed by MALDI-TOF as described in Mahmmod et al. (2018).

The samples were considered positive if ≥ 1 colony of *Staph. aureus* or *Strep. agalactiae* was identified on at least one of the media. If milk samples were contaminated (≥ 3 colony types present), the agar plate would not be considered in the reading, but in this study, one of the media was always useful. Contamination of teat skin samples was not considered due to the origin of the sample. CNS presence was registered for milk and teat skin samples, whereas environmental streptococci, *Enterococcus* and *Aerococcus* spp. were registered as 'other growth' only for milk samples.

3.1.3 Association between the presence of bacteria in teat skin and milk samples

The statistical analyses are described in detail in Manuscript I. Briefly, the association between the presence of bacteria on teat skin and an IMI with the same udder pathogen was assessed in a logistic regression model. Separate models were built for each pathogen (*Staph. aureus* or *Strep. agalactiae*) and test (BC or PCR). The outcome was the presence of *Staph. aureus* or *Strep. agalactiae* in the milk sample (dichotomised) and the explanatory variable of main interest was the presence of the same bacteria in the corresponding teat skin sample (dichotomised). Other categorical variables (parity, days in milk (DIM), SCC at last milk recording, time since last milking, hyperkeratosis score, and various other pathogens detected in milk and teat skin samples) were included as explanatory variables. The analyses were carried out using the Glimmix procedure in SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

3.1.4 Evaluation of test performance using Latent Class Analysis (LCA)

Details on the statistical analyses are described in Manuscript II. As there is no perfect standard for identifying true IMI cases and teat skin colonisation, we evaluated the test performance of BC and PCR on milk and teat skin samples in a Bayesian LCA model using two populations each with a different prevalence (Branscum et al., 2005). The model was implemented in the freeware program OpenBUGS, version 3.2.3, rev. 1012 (Thomas et al., 2006). The population was stratified

based on robot type, which resulted in two populations, each with a different prevalence, as required by the methodology. Test performance was evaluated using both informative and non-informative priors (Manuscript II, Table 1).

3.2 Sampling activity B

This sampling aimed to support investigations on shedding patterns for *Staph. aureus* and *Strep. agalactiae*. Furthermore, the data should be used to compare the different tests available to farmers and veterinarians to improve the interpretation of different test results, which often is a challenge for farmers and herd veterinarians. We therefore investigated diagnostic test patterns and combined quantitative and qualitative analyses, which are also presented in Manuscript III.

3.2.1 Diagnostic test patterns

The first part of this sampling activity was aimed at illustrating the diagnostic test patterns of naturally infected quarters by investigating the quarters over 21 days with three different tests (BC, PCR and SCC).

The aim was to include a herd with a milking parlour where milking took place twice a day and with a high number of cows or quarters with *Strep. agalactiae* infection. We suspected that *Staph. aureus* infections would be more common than *Strep. agalactiae* infections, and a low bulk tank PCR Ct value for *Strep. agalactiae* was therefore a selection criterion for herds. Furthermore, the herd had to be no more than a 1-hour drive away from the commercial laboratory (Eurofins Steins, Vejen, Denmark) to enable same-day BC of the milk samples. The sampling and laboratory work was carried out from June to September 2015.

We aimed to investigate 15 quarters with *Staph. aureus* and 15 quarters with *Strep. agalactiae* from 30 different cows, as it was considered feasible to handle this number of daily samples in the laboratory in addition to sampling and reading plates from the day before. Furthermore, the goal was that there should be no other major pathogens present in the selected quarters. However, it was challenging to find infected quarters that fulfilled those criteria, and despite a low bulk tank Ct value for *Strep. agalactiae* (Ct = 17), the prevalence of infected cows was too low to meet the planned number of quarters. Therefore, another herd was included to reach the planned number of quarters to be investigated, and we included several quarters per cow to reach the target, with some quarters also containing other major pathogens (primarily *Strep. uberis*). No quarters with both *Staph. aureus* and *Strep. agalactiae* were included.

Screening

The screening was carried out on milk samples from quarters, pooled into composite milk samples and tested by PCR (Mastit4, DNA Diagnostic). We used a cut-off Ct value < 40 for *Strep. agalactiae* and Ct value ≤ 37 for *Staph. aureus* to include as many *Strep. agalactiae*-positive cows and quarters as possible. The positive samples from the cows were then analysed at quarter level, and quarters were selected based on the same cut-offs as for cow-level samples. The PCR test kit used for the entire study contained primers for *Staph. aureus, Strep. agalactiae, Strep. uberis, Strep. dysgalactiae* and β-lactamase (Test kit named Mastit4AL).

21-day study

The selected quarters were investigated daily with an aseptically collected milk sample taken just before the cluster was attached and after routine preparation by the farm staff. A sample of 40 to 50 mL was collected and split into three samples at the laboratory: 1) a bronopol-preserved sample for SCC, 2) a bronopol-preserved sample for PCR and 3) a non-preserved sample for BC. The samples were cooled on ice until they arrived at the laboratory within 4 hours (milking time + transport).

The selected cows remained in their normal milking group and were marked with leg bands for easy identification. The farmers were asked not to treat the selected cows without discussing it with me, as antibiotic treatment would exclude the cows from the study. Clinically sick animals were exempt from this strategy, but would still have to be excluded. I collected all milk samples and was therefore present every day during the study period. It was therefore possible to identify if cows were treated with antibiotics, as they would then be marked and milked into buckets.

Laboratory procedures

The SCC, PCR and BC analyses were carried out at Eurofins Steins Laboratory (Vejen, Denmark). The SCC (Fossomatic 5000, Foss, Hillerød, Denmark) and PCR (Mastit4AL) tests were processed and performed through the Danish milk recording system at quarter level. The samples for PCR were delivered directly to the laboratory where the PCR was performed and were therefore not at risk of contamination or mix-up with the milk samples in the routine process. The BC was carried out by me, according to NMC recommendations (NMC, 2004), which are also followed by most Danish herd veterinarians, by streaking 0.01 mL of milk on an aesculin blood agar (Statens Serum Institut, Copenhagen, Denmark). The approximate cfu count was based on counting up to 300 cfu, corresponding to 30,000 cfu/mL. The lower detection limit was 100 cfu/mL.

During the first 3 days of sampling, the colonies were identified based on morphology and results of a latex agglutination test. Furthermore, a representative colony from each quarter was submitted to MALDI-TOF to confirm the identification. Colonies were easily recognised on the following days based on morphology (see Sampling A) and pictures from the previous days. Contamination of samples (defined by ≥ 3 colony types on the plate) was noted, but the presence and approximate cfu of *Staph. aureus* and *Strep. agalactiae* were still recorded. Contaminated samples were included because it was not possible to obtain a new sample on the same day. Quarters that were culture negative after being culture positive were re-cultured after freezing. Furthermore, these samples were re-cultured with 0.05 and 0.1 mL of milk to increase the Se of BC, but the result of the BC did not change.

3.2.2 Expert analysis

The daily test results of BC, PCR and SCC from the study on diagnostic test patterns were graphed at quarter level, representing 40 quarter profiles (Manuscript III, Supplemental Figure S2).

The aim of conducting an expert analysis was to base the interpretation of diagnostic test patterns from the 21-day study (quarter profiles) on a broader perception of IMI. We needed the expert-perceived infection status of each quarter on each day to estimate the test performance for BC, PCR and SCC for the daily samples.

An overview of this process is shown in Figure 3.1.

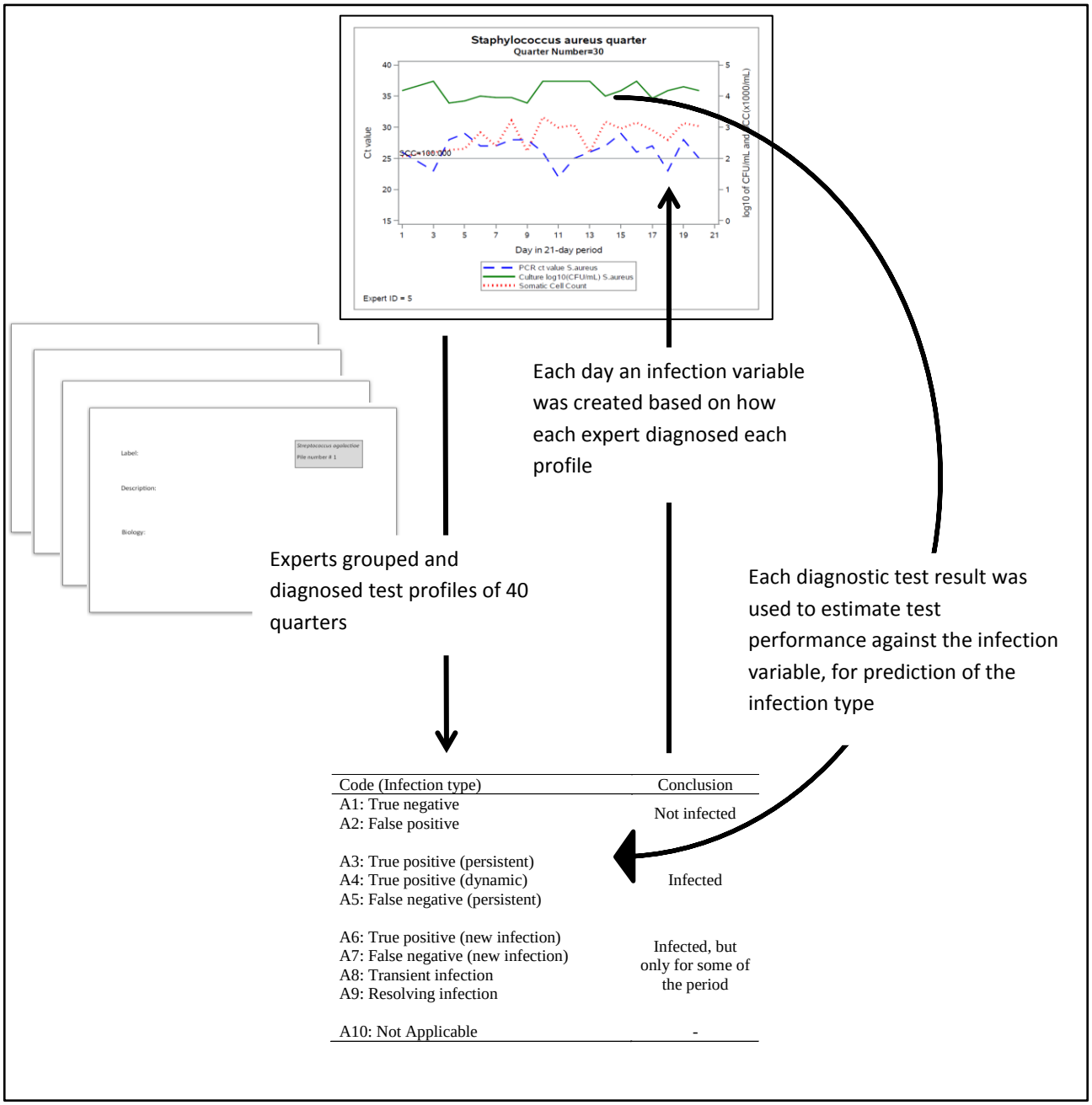


Figure 3.1: Overview of the process using experts' diagnoses of quarter profiles to estimate the test performance of bacterial culture, polymerase chain reaction (PCR) and somatic cell count

At the European Mastitis Research Workers Conference 2017 in Copenhagen, 30 participants assessed the diagnostic test patterns (profiles) of the 40 quarters that were investigated during the 21-day study. The participants were considered to be mastitis experts, with the minimum requirement that they worked with (bovine and/or ovine) mastitis research. A similar set-up and some of the same participants were also used in another expert-based study on diagnosing IMI

(Andersen et al., 2010). The experts were briefly introduced to the origin of the profiles and were provided with small cards on which the profiles were printed. The experts were also given four envelopes marked with *Staph. aureus* and four envelopes marked with *Strep. agalactiae*. The experts were then asked to group the profiles into a maximum of four groups per pathogen, insert the profiles into the envelopes and describe the patterns, explain the underlying biology and make a diagnosis or label for each group.

As a pilot study for calculating test performance based on expert opinion of infection status, we asked two in-house mastitis researchers to group and classify the quarter profiles based on the same instructions that the 30 experts were given. The grouping and descriptions from these researchers were interpreted (by the first author) and assigned to a group of quarters considered either infected or not infected. The probability that BC, PCR and SCC correctly identified a profile on any given day was calculated as the sensitivity (quarters considered infected) or specificity (quarters considered not infected). The pilot study was used to inform the follow-up assessment, but otherwise the two researchers statements were not included.

Statements from the 30 other experts were transcribed and read by two of the authors of Manuscript III (including the first author). A codebook was developed based on the statements. This codebook was intended to condense the explanations in the statements to infection types. The entire codebook is given in Supplemental Table S1 of Manuscript III. The expert statements were then coded by the two authors who developed the codebook, and a third author of Manuscript III. In cases of disagreement among the coders, statements were discussed until a final code was assigned to each group of profiles. An example of coded expert statements is shown in Figure 3.2.

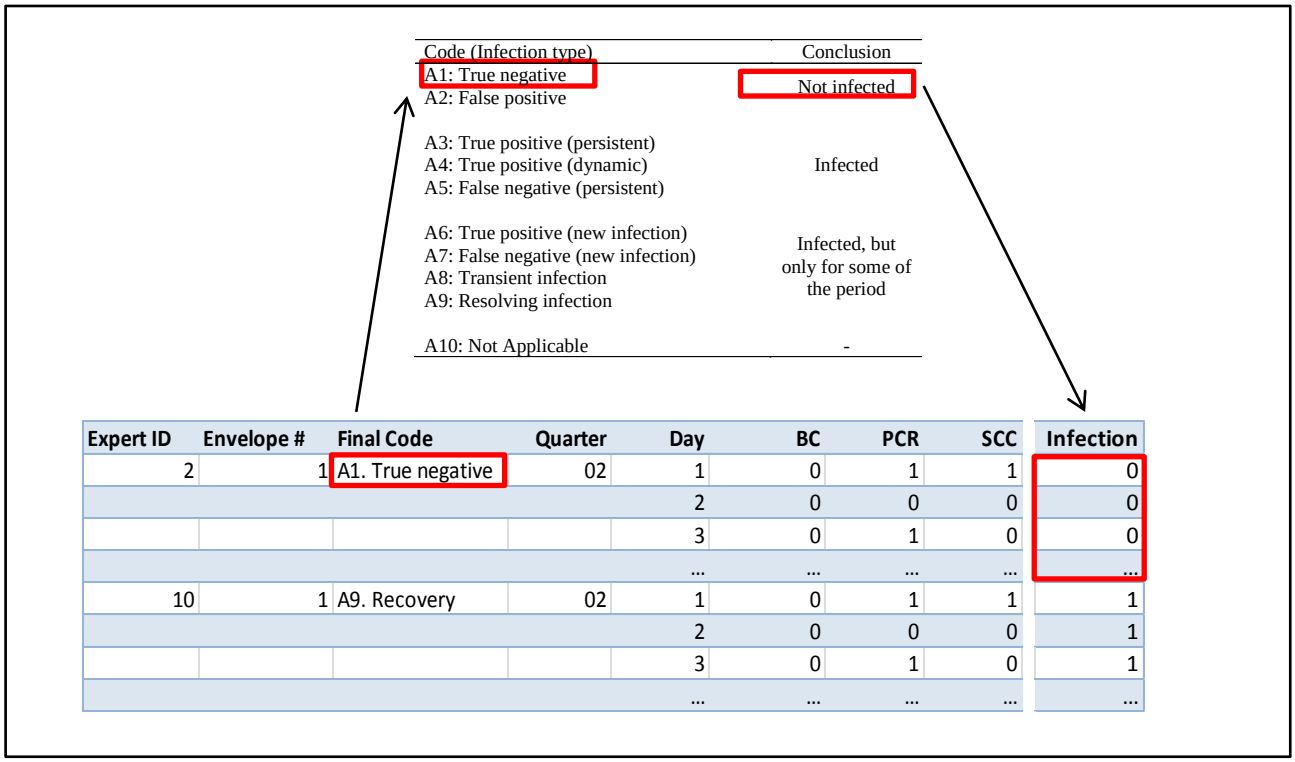
Expert ID	Envelope #	Label	Description	Biology	LS Code	APS Code	SSN Code	Final Code
1	1	Chronic infections/subclinical mastitis	Chronic shedder always positive	Chronic infection well spread in the udder	A3. Persistent.	A3. Persistent.	A3. Persistent.	A3
1	2	Chronic infections/subclinical mastitis	Intermittent shedding with varying frequency	Could be low grade infection spreading through the udder or with genotype specific virulence factors	A4. Dynamic.	A4. Dynamic.	A4. Dynamic.	A4
1	3	Healthy	No infection, low SCC		A1. True negative.	A1. True negative.	A1. True negative.	A1
1	4	Transient infection (probably)	Short-lasting peak	Quick inf. Reaction to inf. Agent and then most likely killed	A8. Transient.	A8. Transient.	A8. Transient.	A8

Figure 3.2: Example of a single expert's description of four *Staphylococcus aureus* groups (columns 3 to 5) coded by three authors (LS, APS and SSN, columns 6 to 8) and assigned a final code (column 9).

3.2.3 Evaluation of test performance based on expert-assigned infection groups

Within each envelope (group of profiles) that was coded as described in the previous section, there were a number of quarter profiles assigned by each expert. The quarters assigned to each of the groups were individual to the expert. A dataset of 30 experts × 40 quarters × 21 days was

therefore created. Each test-day for each quarter was assigned an infection status based on the code assigned to each expert statement for each profile. A schematic example of this process is shown in Figure 3.3. Dichotomised variables of all three tests were created: the PCR test was considered positive at Ct ≤ 37, the BC was considered positive at ≥ 1 cfu/0.01 mL (100 cfu/mL), and SCC was considered positive at 100,000 cells/mL. The infection variable was subsequently used to estimate the test Se and Sp of PCR, BC and SCC, while our understanding (decoded assessment) of the test-day infection status based on expert opinion was used as a reference. This was done by including the infection variable as the outcome variable in logistic regression models, where the test result of BC, PCR or SCC was included as a fixed effect together with the different infection types. Beside estimation of the crude estimates, quarter ID and expert were included as random effects. Due to under-dispersion and lack of model fit, it was necessary to include only a random sample of observations per expert per quarter for some models. For *Strep. agalactiae,* all observations were included in the model with BC, 11 observations per expert per quarter were included for PCR, and only one observation per expert per quarter was included for SCC. For all *Staph. aureus* models, model fit was only achieved with one randomly selected observation per expert per quarter. In addition to these estimates, crude estimates of the Se and Sp of each test were calculated using all observations and no random statements.



Expert ID	Envelope #	Final Code	Quarter	Day	BC	PCR	SCC	Infection
2	1	A1. True negative	02	1	0	1	1	0
				2	0	0	0	0
				3	0	1	0	0
				...	...	...	...	...
10	1	A9. Recovery	02	1	0	1	1	1
				2	0	0	0	1
				3	0	1	0	1
				...	...	...	...	...

Figure 3.3: Example of how the infection variable was assigned for each quarter and each day, based on the code assigned to each expert's grouping of quarter profiles

3.2.4 Multilocus Sequence Typing (MLST)

Quarters were tracked to assess whether the same sequence type (ST) of bacteria was present over the 21 days, and in order to relate a shedding pattern to a specific ST, isolates from two different days (beginning and end of the 21 days) of each of these quarters were sent to DTU-VET for full genome sequencing and MLST. This was only possible in quarters where *Staph. aureus* or *Strep. agalactiae* had been cultured, not in PCR positive samples. The current PhD project only included MLST results. The procedure is described in Ronco et al. (2018).

3.3 Danish Cattle Database

Basic demographic characteristics of the herds and animals included were extracted from the Danish Cattle Database. The data included results of the annual BTM PCR for *Strep. agalactiae* surveillance, the number of cows and AMS type. Information about parity and DIM was summarised at cow level for the included animals, and the SCC results of the latest milk recording were used to select animals in sampling activity A.

4 Results

4.1 Descriptive results from Sampling activity A: Teat skin and milk samples

Data originating from Sampling activity A were used to investigate the association between pathogens present on the teat skin and in milk from the same quarter. Furthermore, PCR and BC were compared to assess test performance on quarter-level milk samples as well as teat skin samples.

Herd characteristics and the proportion of selected cows from the high-SCC cows are shown in Table 4.1. The day of sampling was between 5 and 33 days after the last milk recording, so the SCC recordings used for selection were of different ages.

A total of 300 cows were included in the study, resulting in 1,142 quarters sampled for BC (58 quarters were dry). Of these, 287 were lactating right-rear quarters submitted for PCR analysis. The proportion of milk and teat skin samples positive for *Staph. aureus* and *Strep. agalactiae* varied among herds (Table 4.1.) with both BC and PCR. Detailed cross tabulations of the data including *P*-values from univariable statistics on the association between *Staph. aureus* or *Strep. agalactiae* on teat skin and in milk can be found in Table 2 (BC) and Table 4 (PCR) in Manuscript I.

The overall proportion of positive *Staph. aureus* IMI quarters found using BC was 8.1% (93/1,142), whereas using PCR, this figure was 10% (29/287). *Staph. aureus* was detected on teat skin from 6.6% (75/1,142) and 16% (45/287) of the teats using BC and PCR, respectively.

Strep. agalactiae was detected in 7.4% (84/1,142) and 14% (40/287) of the quarters when using BC and PCR, respectively. For teat skin samples, the proportion of positive teats was 0.35% (4/1,142) and 18% (51/287) as detected by BC and PCR, respectively.

Table 4.1: Herd characteristics, demographic information of sampled cows and prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* in milk and teat skin samples at herd level, as detected by bacterial culture (BC) and polymerase chain reaction (PCR)

Herd	H1	H2	H3	H4	H5	H6	H7	H8
Herd size[1]	267	198	344	298	218	247	333	244
Type of robot (number)	Lely A4 (4)	Lely A2 (3)	Lely A2 (7)	Lely A3 (5)	Lely A2 (4)	Lely A3 (4)	DeLaval (6)	DeLaval (4)
Milk production[2]	10,973	11,098	10,733	11,412	9,024	11,701	11,909	11,020
SCC[3]	183,000	211,000	216,000	199,000	255,000	252,000	192,000	338,000
Breed[4]	100	91	93	83	63	100	98	98
Daily robot cleaning[5]	2 × AW 2 × HPW Brushes in chlorine	2 × AW 2 × HPW	2 × AW 2 × HPW Brushes in washing machine	3 × AW 2 × soap + brush + water Brushes in chlorine	2 × AW 2 × foam + water Brushes in acid	3 × AW 2 × HPW	2 × AW 1-2 × HPW soap + water	2 × AW 1-2 × HPW
Post-milking teat disinfection	0.3% iodine	0.3% iodine	1% lactic acid	0.75% iodine	0.3% iodine	0.3% iodine	0.15% iodine	0.15% iodine
Addition to chopped straw in beds	Hydrated lime	Ökosan GFR[e]	Sanibed[f]	Limestone	Hydrated lime	Basic Strømiddel Destek[g]	Limestone	Destek CombiRenh
Ct value *Strep. agalactiae* bulk milk (×3)	30, 40, 23	25, 32, 22	29, 26, 25	25, 23, 21	26, 24, 20	25, 28, 40	24, 25, 24	24, 21, 22
Ct value *Staph. aureus* bulk milk (×3)	32, 29, 30	25, 40, 31	40, 33, 40	40, 21, 28	32, 29, 28	35, 26, 40	25, 24, 29	31, 28, 27
No. of cows with high SCC[6]	43	43	74	60	49	59	50	79
No. of cows sampled (%)[7]	29 (67)	37 (86)	39 (53)	38 (63)	39 (80)	40 (68)	38 (76)	40 (51)
Median parity of sampled cows	2	2	2	2	2	3	3	3
Median DIM of sampled cows	210	168	182	137	141	158	200	243
Median SCC at last milk recording of sampled cows (cells/mL)	305,000	554,000	512,000	564,000	710,000	381,000	622,000	515,000

No. of quarters sampled for BC	111	145	148	144	149	154	141	150
No. of quarters positive for *Staph. aureus* on teat skin using BC (%)[8]	1 (0.9)	1 (0.7)	14 (9.5)	0 (0.0)	14 (9.4)	10 (6.5)	25 (17.7)	10 (6.7)
No. of quarters positive for *Staph. aureus* in milk using BC (%)[8]	18 (16.2)	4 (2.8)	3 (2.0)	3 (2.1)	3 (2.0)	2 (1.3)	8 (5.7)	52 (34.7)
No. of quarters positive for *Strep. agalactiae* on teat skin using BC (%)[8]	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	1(0.7)
No. of quarters positive for *Strep. agalactiae* in milk using BC (%)[8]	1 (0.9)	0 (0.0)	10 (6.8)	10 (6.9)	17 (11.4)	3 (2.0)	29 (20.6)	14 (9.3)
No. of quarters sampled for PCR	28	36	38	35	37	40	34	39
No. of quarters positive for *Staph. aureus* on teat skin using PCR (%)[8]	5 (17.9)	16 (44.4)	5 (13.2)	0 (0.0)	3 (8.1)	11 (27.5)	1 (2.9)	1 (2.6)
No. of quarters positive for *Staph. aureus* in milk using PCR (%)[8]	6 (21.4)	2 (5.6)	1 (2.6)	0 (0.0)	2 (5.4)	1 (2.5)	3 (8.8)	14 (35.9)
No. of quarters positive for *Strep. agalactiae* on teat skin using PCR (%)[8]	1 (3.6)	11 (30.6)	3 (7.9)	15 (42.9)	4 (10.8)	14 (35.0)	2 (5.9)	1 (2.6)
No. of quarters positive for *Strep. agalactiae* in milk using PCR (%)[8]	1 (3.6)	0 (0.0)	5 (13.2)	18 (51.4)	3 (8.1)	3 (7.5)	7 (20.6)	3 (7.7)

[1] Includes both lactating and dry cows; [2] Estimated kg ECM/cow per year; [3] Cells/mL: Geometric mean of bulk tank SCC within last 3 months; [4] Danish Holstein (%); [5] Information obtained from the farmer: AW = automatic wash, HPW = high pressure washer; [6] SCC > 200,000 cells/mL at last milk recording; [7] % of cows with somatic cell count > 200,000 cells/mL [8] % of quarters sampled in herd

[a] pH < 3, hydrogen peroxide, peracetic acid and acetic acid; [b] pH = 1, peracetic acid, acetic acid and hydrogen peroxide; [c] pH < 1, peracetic acid, hydrogen peroxide and acetic acid; [d] pH = 0.5, hydrogen peroxide and peracetic acid; [e] pH = 12, calcium compounds; [f] pH = 2.9, salicylic acid; [g] pH = 8, tosylchloramide sodium; [h] pH = 8-10, tosylchloramide sodium

4.2 Association between the presence of bacteria on teat skin and in milk

The results from this study were taken from Sampling activity A, and are presented in Manuscript I. Simple cross tabulations of positive milk and teat skin samples detected by PCR or BC are shown in Table 4.2 with the crude odds ratio for the association between the presence of pathogens on teat skin and in milk.

For *Staph. aureus* detected by PCR, the explanatory variable of primary interest (teat skin colonisation) did not meet the inclusion criterion ($P < 0.20$ in the univariable analysis), thus no multivariable analysis was carried out (Manuscript I). For *Staph. aureus* detected by BC, the odds of *Staph. aureus* IMI were 7.8 [95% CI; 2.9 - 20.6] times higher if the teat skin was colonised with *Staph. aureus*. In contrast, for *Strep. agalactiae* detected by BC, the number of positive teat skin samples was too low to carry out statistical analysis. Therefore, no odds ratio was calculated using the *Strep. agalactiae* BC data. For *Strep. agalactiae* detected by PCR, the odds of *Strep. agalactiae* IMI were 3.8 [95% CI; 1.4 - 10.1] times higher if *Strep. agalactiae* was detected on teat skin compared to a quarter with a negative teat skin sample.

The results of the final multivariable analyses are shown in Manuscript I, Table 3 (*Staph. aureus* BC) and Table 5 (*Strep. agalactiae* PCR). The following variables were included in the final *Staph. aureus* BC model: herd (H1-H8), hyperkeratosis (Score 1-4) and other growth in milk (dichotomous variable of the presence of pathogens other than *Staph. aureus, Strep. agalactiae* and CNS). The odds of *Staph. aureus* IMI increased with increasing hyperkeratosis score, while the odds decreased when other pathogens were present in the milk. Hyperkeratosis (score 1-4), parity (1, 2 or ≥3), SCC (200-399, 400-999 or ≥1,000 ×1,000 cells/mL) and CNS on teat skin (dichotomous) were included in the final *Strep. agalactiae* PCR model. The odds of *Strep. agalactiae* IMI increased with increasing hyperkeratosis score, parity group, SCC group and when CNS was detected on teat skin.

4.3 Test performance of BC and PCR for teat skin and milk samples using LCA

The results from this study are presented in Manuscript II. The LCA was carried out on BC and PCR data from Sampling activity A, using the same 287 quarters used in the association study (PCR data) in Manuscript I. The cross-tabulated results from BC and PCR for *Staph. aureus* and *Strep. agalactiae* stratified by the two populations (robot type) are shown in Manuscript II, Table 2.

The estimated Se and Sp of BC and PCR for detection of *Staph. aureus* and *Strep. agalactiae* on teat skin and in milk are given in Manuscript II, Table 3. To give an overview, the Se and Sp estimates based on informative as well as non-informative priors are plotted in Figure 4.1 for *Staph. aureus* and *Strep. agalactiae*.

Table 4.2: Cross tabulations of *Staphylococcus aureus* and *Streptococcus agalactiae* in milk and teat skin samples of 287 quarters tested by polymerase chain reaction (PCR) and 1,142 quarters tested by bacterial culture

Staphylococcus aureus **PCR**				
		Milk		
		0	1	Total
Teat skin	0	218 (89%)	27 (11%)	245 (100%)
	1	40 (95%)	2 (5%)	42 (100%)
	Total	258	29	287

Odds ratio = 0.4 [95% CI; 0.1 – 1.8]

Staphylococcus aureus **Bacterial culture**				
		Milk		
		0	1	Total
Teat skin	0	989 (93%)	78 (7%)	1,067 (100%)
	1	60 (80%)	15 (20%)	75 (100%)
	Total	1,049	93	1,142

Odds ratio = 3.2 [95% CI; 1.7 - 5.8]

Streptococcus agalactiae **PCR**				
		Milk		
		0	1	Total
Teat skin	0	212 (90%)	24 (10%)	236 (100%)
	1	35 (69%)	16 (31%)	51 (100%)
	Total	247	40	287

Odds ratio = 4.0 [95% CI; 2.0 - 8.4]

Streptococcus agalactiae **Bacterial culture**				
		Milk		
		0	1	Total
Teat skin	0	1,057 (93%)	81 (7%)	1,138 (100%)
	1	1 (25%)	3 (75%)	4 (100%)
	Total	247	40	1,142

Odds ratio = 39.2 [95% CI; 4.0 – 380.6]

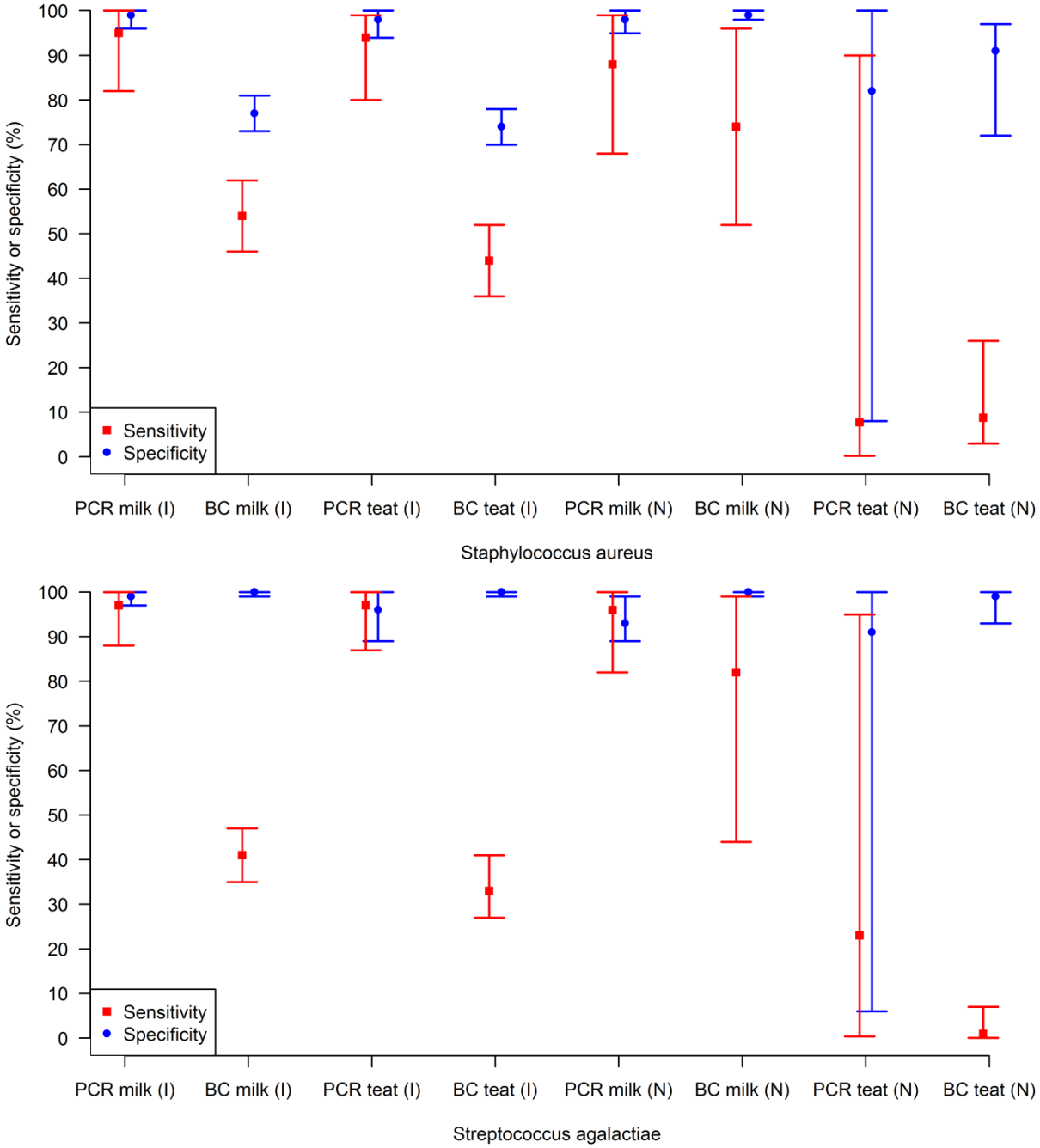


Figure 4.1: Estimated sensitivity (red) and specificity (blue) including 95% posterior credible intervals of polymerase chain reaction (PCR) and bacterial culture (BC) for *Staphylococcus aureus* (top) and *Streptococcus agalactiae* (bottom) in milk and teat skin samples using informative (I) and non-informative (N) priors

4.4 Descriptive results from Sampling activity B: Diagnostic test patterns

Data originating from Sampling activity B were used to investigate diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae* in naturally infected quarters. The quarters were selected based on a single positive PCR test at cow or quarter level. The results from screening 589 cows from two herds are shown in Table 4.3.

Table 4.3: Selection of quarters to be investigated in the 21-day study showing the proportion of *Staphylococcus aureus*- and *Streptococcus agalactiae*-positive cows and quarters based on a single polymerase chain reaction (PCR) test using cut-offs of Ct ≤ 37 and Ct < 40 for *Staph. aureus* and *Strep. agalactiae*, respectively

Herd	Pathogen	PCR Ct value in bulk tank milk	Cows tested by composite milk samples	Cows positive in composite milk samples (% of total cows tested)	Quarters positive at screening (% of cows × 4 quarters)
Herd 1	*Staph. aureus*	36	112	9 (8.0)	9 (25.0)
	Strep. agalactiae	17		5 (4.5)	9 (45.0)
Herd 2	*Staph. aureus*	32	487	14 (2.9)	15 (26.8)
	Strep. agalactiae	29		6 (1.2)	7 (29.2)
Total			589	34	40

Of the 34 cow-level samples that were tested at quarter level, 40 quarter-level samples from 31 cows were positive and therefore included in the study (one to four quarters per cow). The cows included were between 1st and 5th lactation (median = 2nd). The median DIM was 176 at the date of screening, ranging from 12 to 505 DIM. At day 20 in the study, two cows were dried off, and therefore four quarters were only investigated for 20 days. Test results of ten SCC and seven PCR tests were missing because analyses failed. As a consequence, these test results could not be plotted in the diagnostic test profiles (quarter profiles).

In total, 24 quarters with *Staph. aureus* and 16 quarters with *Strep. agalactiae* were investigated. The diagnostic test profiles of all quarters are illustrated in an alternative setup stratified by pathogen and infection type (see later) in Figures 4.2-4.6. These plots should give an overview of the consistency and variability in test results of the quarters investigated. The graphs (of each quarter) generally overlap, but some variation exists. The resulting variation also means that some cows will be above or below the thresholds. However, there may not be any obvious patterns for the individual, apart from those identified by the experts (see later). In addition, the daily test results of BC, PCR and SCC are plotted at quarter level for all 40 quarters in Manuscript III, Supplemental Figure S2.

Based on PCR, six *Staph. aureus*-positive quarters had a single day when they were also positive for *Strep. agalactiae* (Ct 29 to 31). This was not the case when using BC. One of these quarters was also positive for *Strep. uberis* and *Strep. dysgalactiae*. Three quarters with *Staph. aureus* were constantly positive for *Strep. uberis* (Ct 20 to 32), which was confirmed by BC for two of the quarters. One quarter was constantly positive for *Strep. dysgalactiae* (Ct 20 to 26) and three quarters were occasionally positive (a single day). The constantly positive quarter was also positive using BC, but the occasionally positive quarters were listed as contaminated using BC. Quarters constantly positive for a pathogen other than the target may influence the SCC for that quarter.

Five quarters with *Strep. agalactiae* were occasionally found to be positive for *Staph. aureus* using PCR (Ct 28 to 36). The amount of *Staph. aureus* occasionally detected in quarters with *Strep. agalactiae* using BC was about the same, but this did not correspond to the same quarters or days, indicating that the *Staph. aureus* found was most likely contamination and a low concentration of bacterial DNA. One quarter with *Strep. agalactiae* was found to be constantly positive for *Strep. uberis* using PCR (Ct 21 to 30), which was confirmed by BC. Three quarters each had a single day when *Strep. uberis* was detected, which was most likely contamination. Three *Strep. agalactiae*-positive quarters were occasionally found to be positive for *Strep. dysgalactiae* (Ct > 30) by PCR.

4.5 Infection types based on expert analysis of diagnostic test patterns

Results of this study are presented in Manuscript III. The 30 experts grouped and described the shedding patterns of the 40 quarters investigated in Sampling activity B. The experts grouped the shedding patterns into two to four groups for each pathogen, but for the most part all four groups (envelopes) were used. The codes (infection types) that covered the expert statements and distribution of codes at envelope level are shown in Manuscript III, Table 1. For *Staph. aureus,* 13 of 117 envelopes were coded “not applicable” and were thereby excluded from further analysis. For *Strep. agalactiae,* the number of envelopes excluded was 21 out of 112. Each quarter was assigned an infection type for each expert, based on the expert statements and the codes assigned at envelope level. The distribution of codes at quarter level is shown in Table 4.4 (*Staph. aureus*) and Table 4.5 (*Strep. agalactiae*). The number of statements excluded for each quarter can be inferred from the total number of codes assigned to each quarter. Only four quarters with *Staph. aureus* were included for all 30 experts, meaning that the remaining quarters appeared at least once in an envelope that was assigned the “not applicable” code.

From the distribution of codes to each quarter in Tables 4.4 and 4.5, it appears that some quarters were mainly assigned to certain codes, indicating that the experts agreed on the diagnosis of these quarters. Of the 24 quarters with *Staph. aureus*, four were mainly considered not infected (A1 and A2), 17 were mainly considered persistently infected (A3 and A5), and three were mainly considered to have a dynamic infection (A4). Of the 16 quarters with *Strep. agalactiae*, nine were mainly considered persistently infected, two were considered not infected and five were considered both infected and not infected.

Figures 4.2 to 4.6 present the daily results of PCR, BC and SCC for each of the investigated quarters graphed in groups based on the codes primarily assigned by the experts (Tables 4.4 and 4.5). In the quarters mainly considered infected (Figures 4.2. and 4.3), the Ct value from the PCR test was generally below 30, the approximate log(cfu/mL) was generally above 3, corresponding to 1,000 cfu/mL, and the quarter SCC was generally above 100,000 cells/mL. For *Staph. aureus* in particular, there were cases of occasionally negative PCR test results (Ct = 40), which may have been considered false-negative test results by the experts.

The three *Staph. aureus*-positive quarters diagnosed with a dynamic infection (Figure 4.4) shifted between positive and negative test results for both PCR and BC, yet the two tests rarely agreed. Five quarters with *Strep. agalactiae* showed variation in test results, especially with PCR and BC, and were coded both infected and not infected (Figure 4.5). The quarters mainly considered not infected (Figure 4.6) were, in most cases, found to be negative using BC and PCR, and had SCC < 100,000 cells/mL. However, occasionally positive PCR test results were seen in three quarters with *Staph. aureus*, one also confirmed by BC.

Table 4.4: Distribution of codes for 24 quarters with *Staphylococcus aureus* based on experts' statements

	***Staphylococcus aureus* quarter number**																							
Code	**01** a	**07** a	**08** a	**10** a	**11** b	**12** c	**14** a	**15** a	**17** b	**20** a	**25** a	**26** b	**27** a	**28** a	**29** a	**30** a	**31** a	**33** a	**34** a	**35** c	**36** c	**37** c	**38** a	**39** a	**Total**
A1: True negative[1]					1	6														14	8	14			**43**
A2: False positive[1]	1				1	9														10	10	10			**41**
A3: True positive (persistent)[2]	17	23	23	27			17	23	2	15	9		26	27	21	26	25	15	9				16	14	**335**
A4: True positive (dynamic)[2]	2	3	4	2	21	3	4	3	23	8	14	25	2	1	3	3	1	4	14		1		7	5	**153**
A5: False negative (persistent)[2]	4	3	2	1	1		4	3		4	4		2	2	2	1	2	4	4				4	6	**53**
A6: True positive (new infection)[3]																									**0**
A7: False negative (new infection)[3]																									**0**
A8: Transient infection[3]					2	9			1			1								4	6	4			**27**
A9: Recovery[3]						1														1	1	1			**4**
Total	**24**	**29**	**29**	**30**	**26**	**28**	**25**	**29**	**26**	**27**	**27**	**26**	**30**	**30**	**26**	**30**	**28**	**23**	**27**	**29**	**26**	**29**	**27**	**25**	**656**

[1]A1 and A2 are considered not infected; [2]A3 – A5 are considered infected; [3]A6 – A9 are considered infected for a period over the 21 days; [a] Mainly diagnosed as persistently infected; [b] Mainly diagnosed as a dynamic infection; [c] Mainly diagnosed as not infected

Table 4.5: Distribution of codes for 16 quarters with *Streptococcus agalactiae* based on experts' statements

	***Streptococcus agalactiae* quarter number**																
Code	**02** [b]	**03** [a]	**04** [a]	**05** [a]	**06** [a]	**09** [b]	**13** [a]	**16** [c]	**18** [b]	**19** [a]	**21** [b]	**22** [b]	**23** [a]	**24** [a]	**32** [a]	**40** [c]	**Total**
A1: True negative[1]	4					9		16	2			3				16	**50**
A2: False positive[1]	6					8		6	3			4				6	**33**
A3: True positive (persistent)[2]		20	20	21	21		13		1	12	6	1	20	18	17		**170**
A4: True positive (dynamic)[2]	2	1	1			2	3		3	3	3	4		2	2		**26**
A5: False negative (persistent)[2]	4	6	6	6	6	1	10	1	5	8	7	5	7	7	7	1	**87**
A6: True positive (new infection)[3]	1								1		7	1					**10**
A7: False negative (new infection)[3]											1						**1**
A8: Transient infection[3]						3		2			1					2	**8**
A9: Recovery[3]	3					1			4			3					**11**
Total	**20**	**27**	**27**	**27**	**27**	**24**	**26**	**25**	**19**	**23**	**25**	**21**	**27**	**27**	**26**	**25**	**396**

[1]A1 and A2 are considered not infected; [2]A3 – A5 are considered infected; [3]A6 – A9 are considered infected for a period over the 21 days; [a] Mainly diagnosed as persistent infected; [b] Diagnosed both as infected and not infected; [c] Mainly diagnosed as not infected

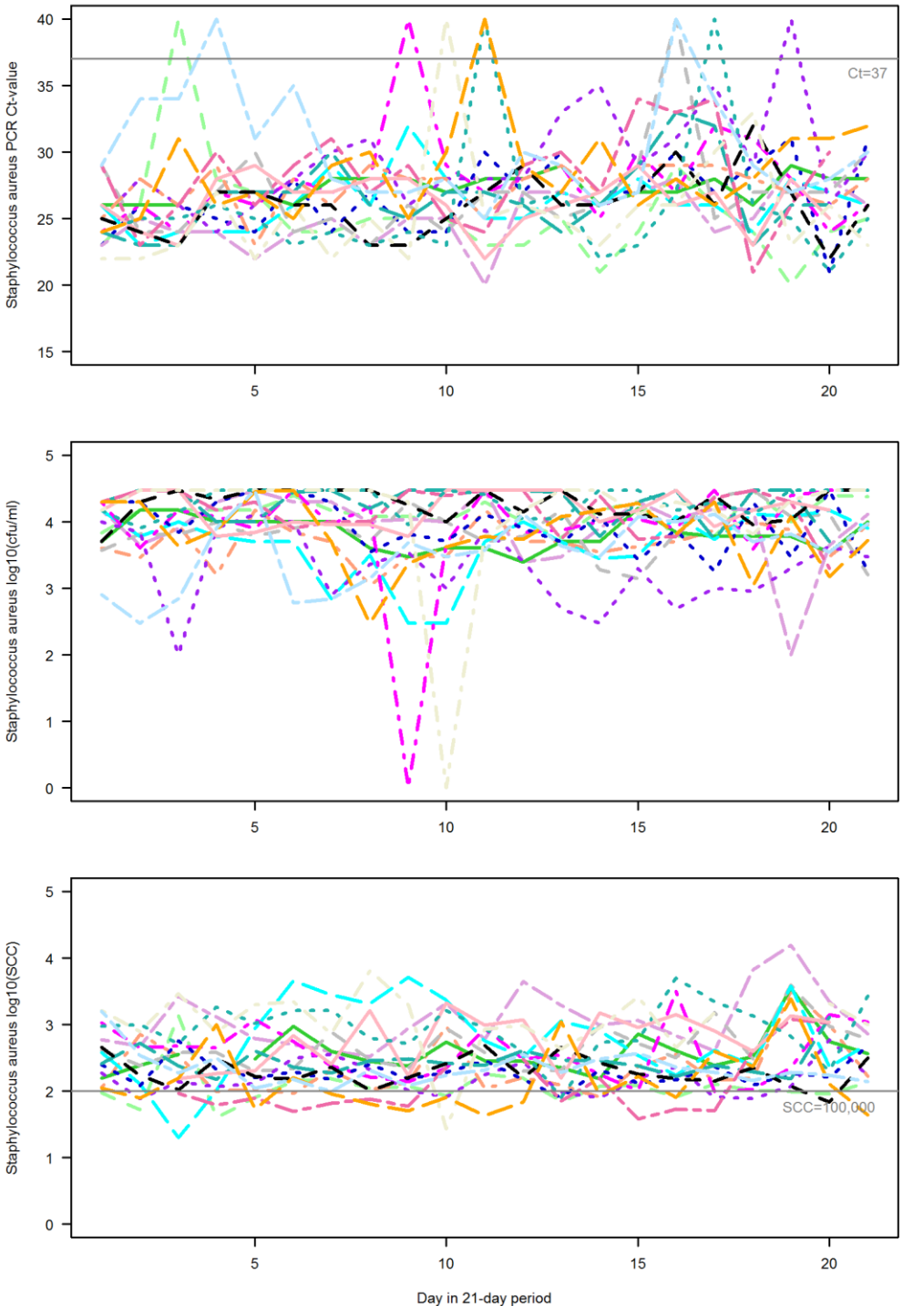


Figure 4.2: 21-day test results of polymerase chain reaction, bacterial culture and somatic cell count for 17 quarters (each colour represents a quarter) with *Staphylococcus aureus,* mainly diagnosed by 30 mastitis experts as persistently infected

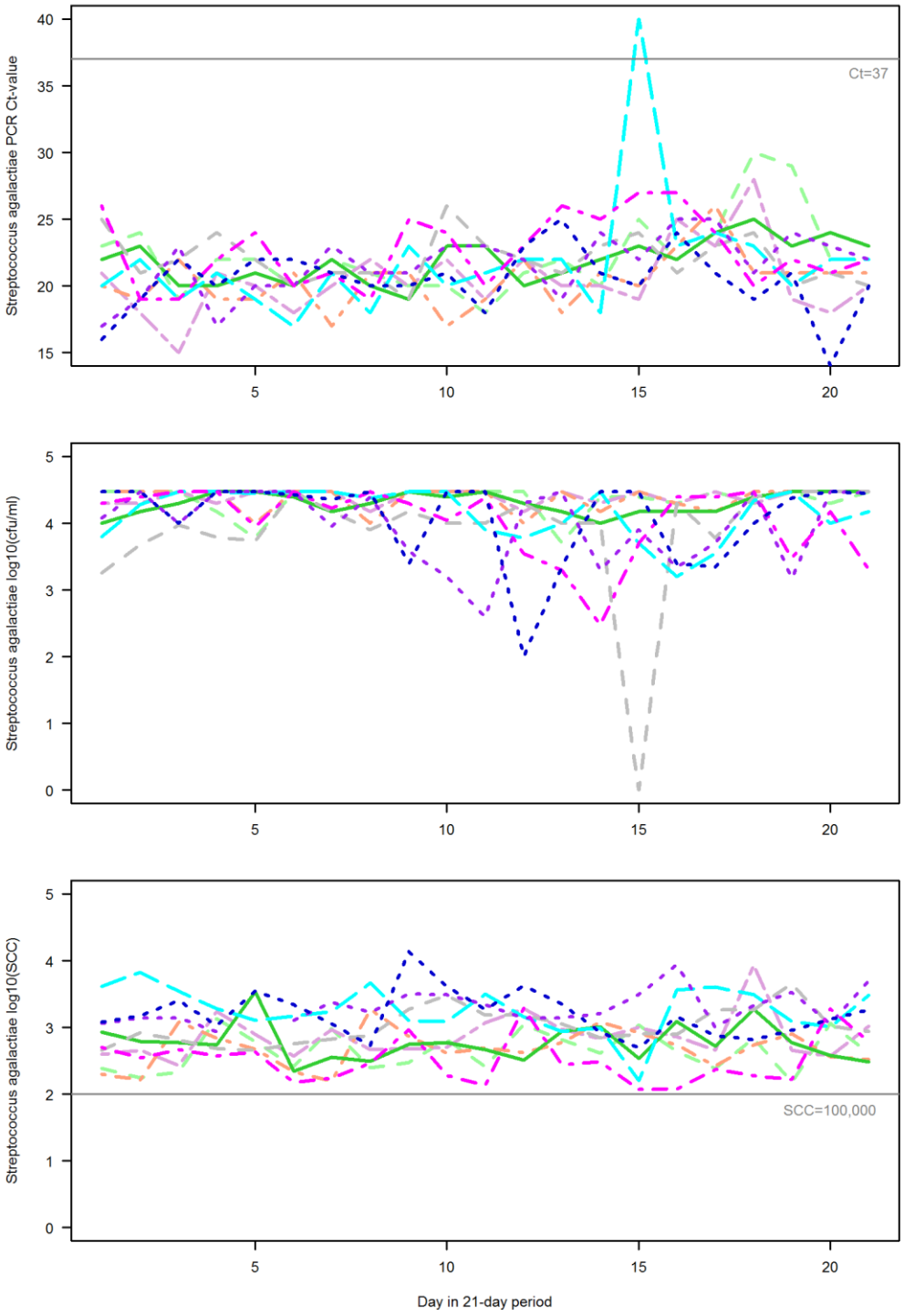


Figure 4.3: 21-day test results of polymerase chain reaction, bacterial culture and somatic cell count for nine quarters (each colour represents a quarter) with *Streptococcus agalactiae,* mainly diagnosed by 30 mastitis experts as persistently infected

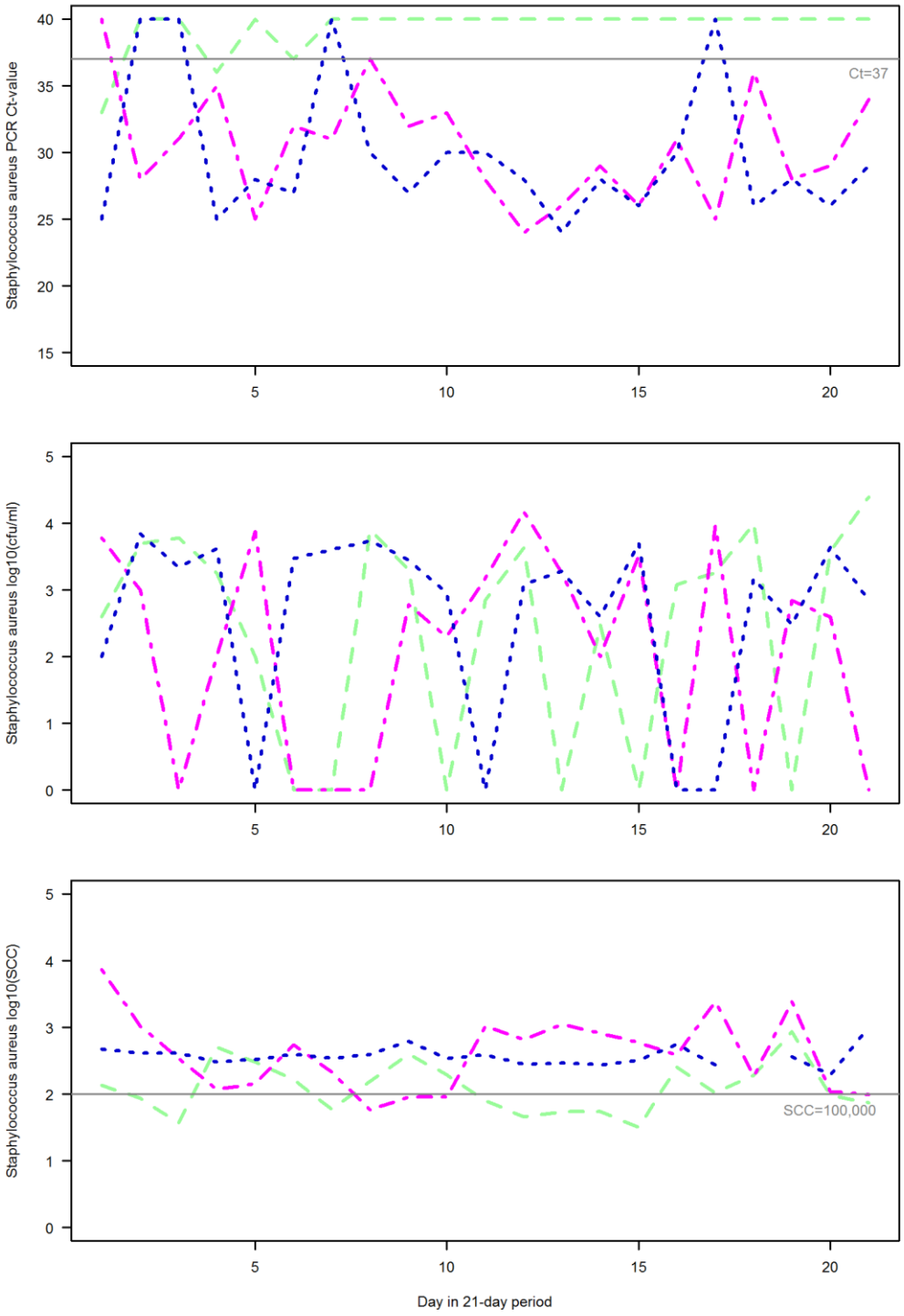


Figure 4.4: 21-day test results of polymerase chain reaction, bacterial culture and somatic cell count for three quarters (each colour represents a quarter) with *Staphylococcus aureus,* mainly diagnosed by 30 mastitis experts as dynamic infected

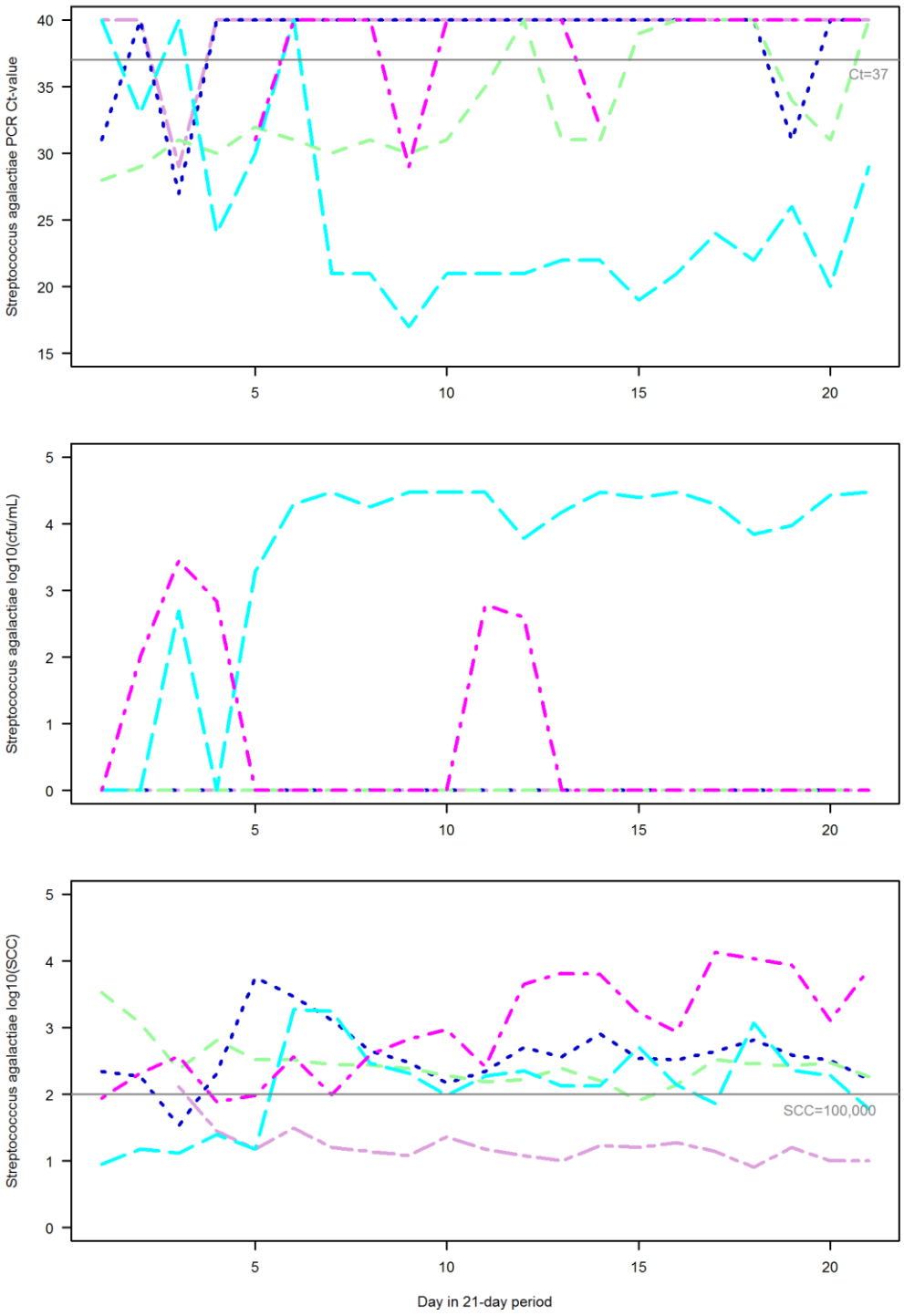


Figure 4.5: 21-day test results of polymerase chain reaction, bacterial culture and somatic cell count for five quarters (each colour represents a quarter) with *Streptococcus agalactiae,* diagnosed by 30 mastitis experts as both infected and not infected

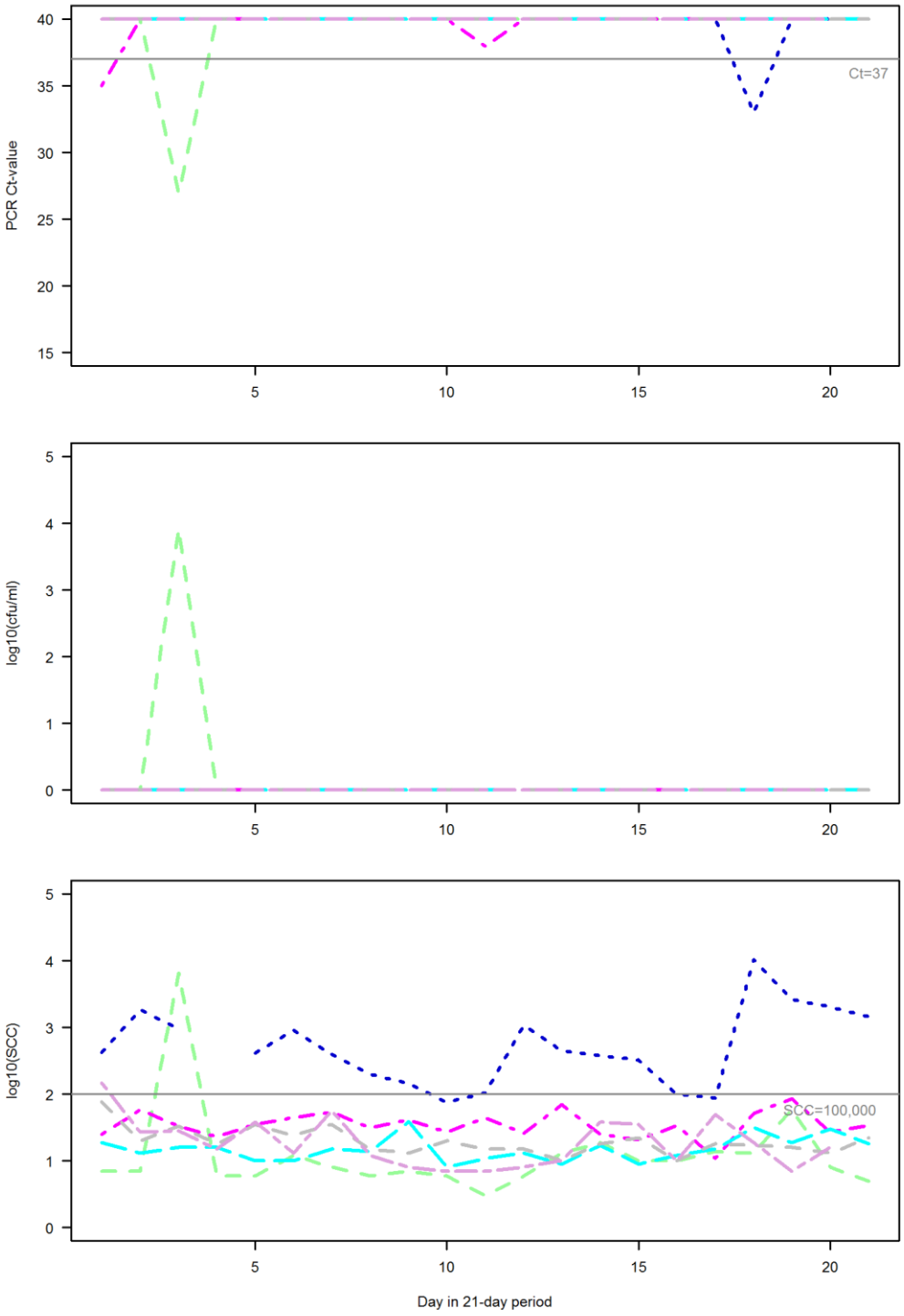


Figure 4.6: 21-day test results of polymerase chain reaction, bacterial culture and somatic cell count for two quarters with *Streptococcus agalactiae* (grey and plum) and four quarters with *Staphylococcus aureus* (green, blue, cyan and magenta), mainly diagnosed by 30 mastitis experts as not infected

4.6 Test performance of BC, PCR and SCC based on expert analysis

Test performance results from Sampling activity B are presented in this section. The test performance of BC, PCR and SCC was evaluated based on two researchers in a pilot study, and on infection type groups created by 30 experts. In both cases, the mastitis researchers and experts set the reference of infection status for each quarter in estimation of the Se and Sp.

4.6.1 Test performance based on test pattern diagnosis by two researchers

These results were to demonstrate how test performance could differ depending on the difference in infection status based on interpretations of diagnostic test patterns. The two mastitis researchers both used all four allowed groups for each pathogen, but used different grouping and diagnoses for the groups. Their diagnoses and the estimated chance of correctly identifying an infected (Se) and non-infected (Sp) quarter are shown in Table 4.6 (*Staph. aureus*) and Table 4.7 (*Strep. agalactiae*). For *Staph. aureus,* the same quarters were diagnosed as infected and not infected by both researchers. The estimated chance of correctly identifying an infected and non-infected quarter was therefore the same for both researchers. In contrast, *Strep. agalactiae* was grouped differently depending on the researcher. Researcher B had a "contaminated" group, which lowered the Sp of BC and in particular PCR, as positive test results were considered contamination. This demonstrates how different diagnoses can be made based on the same test results, depending on the individual who interprets them.

Table 4.6: Probabilities of correctly identifying infected and non-infected quarters with *Staphylococcus aureus* using bacterial culture (BC), polymerase chain reaction (PCR) and somatic cell count (SCC) based on diagnoses by two mastitis researchers

Staph. aureus		Profile[1]	N	BC [95% CI]	PCR [95% CI]	SCC [95% CI]
Infected	Researcher A	Chronic IMI	9	95.4 [92.9;97.1]	91.6 [88.9;94.3]	83.7 [80.1;87.3]
		Uncontrolled IMI	3			
		Error (false negative)	8			
	Researcher B	Chronic	17	95.4 [92.9;97.1]	91.6 [88.9;94.3]	83.7 [80.1;87.3]
		New infection	3			
Non-infected	Researcher A	No IMI	4	98.8 [96.5;100]	96.4 [92.4;100]	77.1 [68.1;86.1]
	Researcher B	Cleared infection	3	98.8 [96.5;100]	96.4 [92.4;100]	77.1 [68.1;86.1]
		Clearing infection	1			

[1] Expert description: Chronic IMI = constant shedding in BC and PCR, Uncontrolled IMI = multiple negative BC and PCR tests, Error = individual (1-2) negative BC or PCR tests, Chronic = constant positive BC and PCR tests, New infection = highly variable BC and PCR, No IMI = negative BC and PCR tests, Cleared infection = negative BC and PCR tests, Clearing infection = few positive BC and PCR tests and decreasing SCC.
The table is modified from Table 2 in Additional scientific work B

Table 4.7: Probabilities of correctly identifying infected and non-infected quarters with *Streptococcus agalactiae* using bacterial culture (BC), polymerase chain reaction (PCR) and somatic cell count (SCC) based on diagnoses by two mastitis researchers' diagnoses

Strep. agalactiae		Profile[1]	N	BC [95% CI]	PCR [95% CI]	SCC [95% CI]
Infected	Researcher A	IMI	7	98.1 [96.2;99.9]	98.1 [96.2;99.9]	96.2 [93.6;98.8]
		Error (false negative)	3			
	Researcher B	Chronic	10	83.7 [79.1;88.3]	91.5 [88.0;95.0]	94.4 [91.6;97.2]
		New infection	2			
Not infected	Researcher A	No IMI	2	96.0 [92.6;99.4]	80.7 [75.2;86.2]	52.4 [43.6;61.2]
		Contaminated	4			
	Researcher B	Cleared infection	2	100 [100;100]	95.2 [90.6;99.8]	73.5 [64.0;83.0]
		Clearing infection	2			

[1]Expert description: IMI = constant shedding in BC and PCR, Error = individual negative BC or PCR tests, Chronic = constant positive BC and PCR tests with the exception of few false positives, New infection = only positive by PCR due to low number of bacteria, No IMI = negative BC and PCR tests, Contaminated = negative BC, variable PCR, Cleared infection = negative BC and PCR tests, Clearing infection = few positive PCR tests and decreasing SCC.
The table is modified from Table 1 in Additional scientific work B

4.6.2 Test performance based on evaluation of shedding patterns by 30 experts

Results from this study are also presented in Manuscript III. For *Staph. aureus,* 13,690 observations (expert-quarter-days) were included representing the test-days from 24 quarters diagnosed by up to 30 experts. For *Strep. agalactiae* 8,291 observations were included representing the test-days of 16 quarters diagnosed by up to 30 experts.

The estimated Se and Sp of different infection types are presented in Figure 4.7 along with the crude estimates and estimates from the pilot study (two researchers). The Se estimates of BC and PCR were generally high with varying Sp. The crude estimates, which were estimated without including expert and quarter as random effects and without taking the different diagnoses into account, have narrow CI due to repetitions in the data. The results from the two researchers are very similar to the crude estimates, especially for *Staph. aureus*, but with wider CI. The test performance was assessed for overall infection, new infection, transient infection and recovery in the logistic regression models. However, some estimates were not applicable due to the lack of data caused by the random sample of observations included in the models to obtain model fit. Furthermore, no quarters with *Staph. aureus* were diagnosed as having new infections, so no estimates could be assessed.

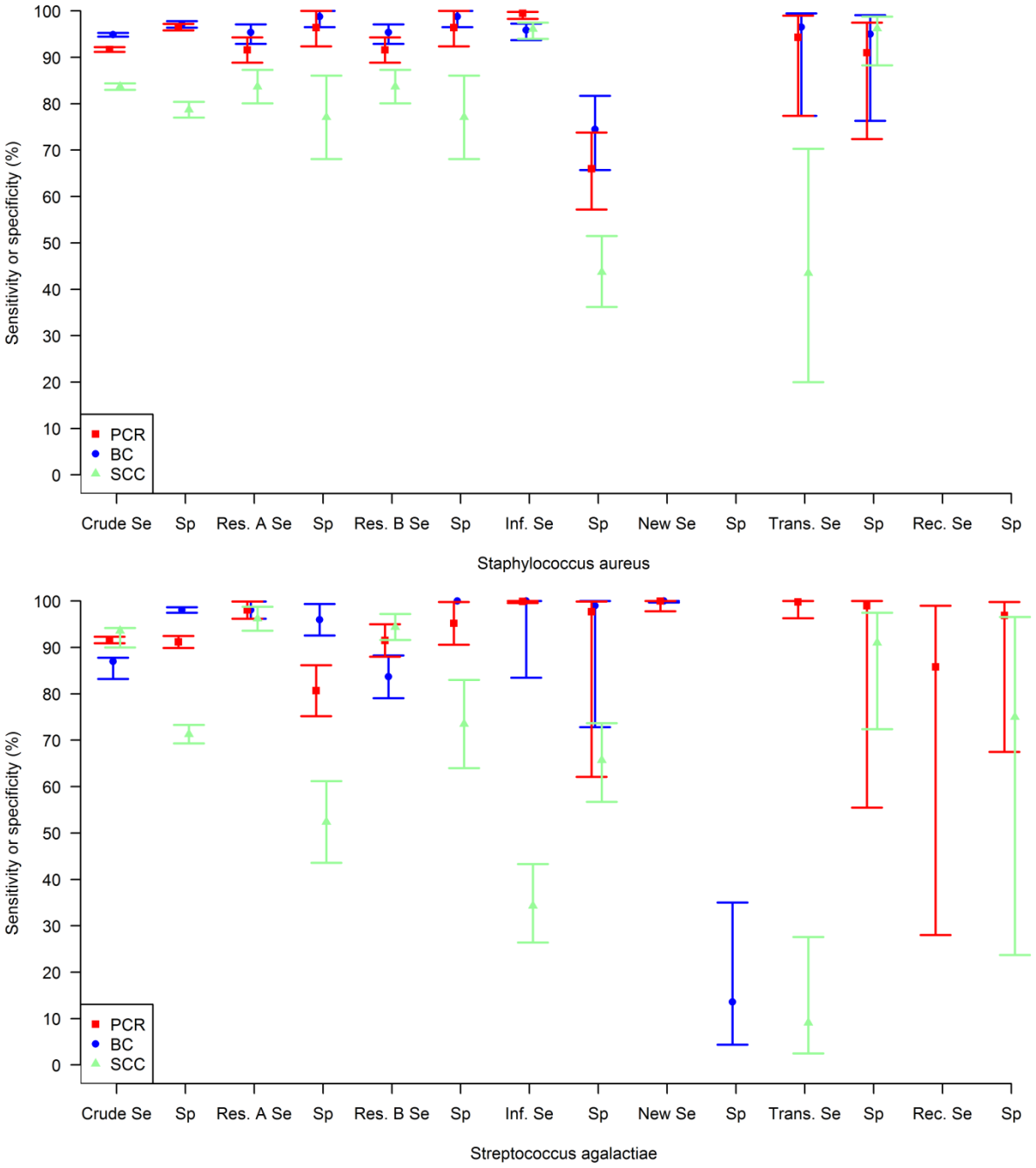


Figure 4.7: Estimated sensitivity (Se) and specificity (Sp) including 95% confidence intervals of PCR (red), bacterial culture (BC, blue) and SCC (green) for detection of *Staphylococcus aureus* (top) or *Streptococcus agalactiae* (bottom) in milk from quarters with different infection types (Inf. = overall infection, New = new infection, Trans. = transient infection, Rec. = recovery) and crude estimates from the expert analysis and Se and Sp calculated based on data from two researchers (Res. A and Res. B)

4.7 Sequence types of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated in Sampling activity B

It was possible to culture *Staph. aureus* at least twice during the 21-day period from 20 of the 24 included quarters with *Staph. aureus*. For *Strep. agalactiae,* ten quarters were cultured. The ST of an early and late sample from the 21-day period are given in Table 4.8. The same ST was identified both early and late in the 21-day period for all quarters. Quarters from the same cows had the same ST. Multiple (three to four) different ST were found for *Staph. aureus* in each herd, whereas a single ST was identified in each herd for *Strep. agalactiae*. There was no obvious association between ST and infection type assigned in the expert analysis (Manuscript III, Supplemental Figure S2).

Table 4.8: Sequence types (ST) of quarters with *Staphylococcus aureus* and *Streptococcus agalactiae* investigated over 21 days with the ST of an early and late sample

Pathogen	Herd	Quarter number[1]	Sequence type (ST)	
			Early sample	Late sample
Staphylococcus aureus	1	01	ST 133	ST 133
		07 + 08	ST 151	ST 151
		10 + 11[a]	NA	ST 45
		14	ST 504	ST 504
		15	ST 504	ST 504
		17[a]	ST 133	ST 133
	2	20	ST 50	ST 50
		25	ST 50	ST 50
		26[a]	ST 71	ST 71
		27	ST 50	ST 50
		28 + 29 + 30	ST 50	ST 50
		31	ST 50	ST 50
		33	ST 50	ST 50
		34	ST 50	ST 50
		38	ST 72	ST 72
		39	ST 72	ST 72
Streptococcus agalactiae	1	03 + 04 + 05 + 06	ST 626	ST 626
		13	ST 626	ST 626
	2	19	ST 8	ST 8
		21	ST 8	ST 8
		23	ST 8	ST 8
		24	ST 8	ST 8
		32	ST 8	ST 8

[1] quarters from the same cow are given in the same row [a] quarter with dynamic *Staph. aureus* infection according to expert-assigned infection type

5 General discussion

The aim of this PhD project was to improve the recommendations for efficient diagnosis and control of contagious mastitis in dairy herds by gaining more knowledge about dynamics of the contagious udder pathogens *Staph. aureus* and *Strep. agalactiae*. Although there are several diagnostic tests to detect udder pathogens, farmers and herd veterinarians still raise questions about the interpretation of their results in practice (i.e. on farm). Furthermore, despite the implementation of control programmes, IMI with *Staph. aureus* and *Strep. agalactiae* still occurs, often with a high or increasing prevalence.

We tend to think that "more diagnostics are always better", but we may fail to consider the quality of the diagnostic results. Results from this project showed that precautions are needed when diagnosing IMI with *Staph. aureus* and *Strep. agalactiae*.

Staph. aureus and *Strep. agalactiae* can be detected (especially when using PCR) on the teat skin of quarters that have and quarters that do not have an IMI with the same pathogen (Manuscript I). Furthermore, there was an association between the presence of pathogens on teat skin and in milk (RQ1 and RQ2), which indicates that the teat skin should be considered a reservoir for contagious udder pathogens. This reservoir should be taken into consideration in future control programmes. However, the proportion of quarters from which *Strep. agalactiae* was isolated on the teat skin using BC was low (0.35%), indicating either that BC is a poor tool for detecting *Strep. agalactiae* on teat skin, or that *Strep. agalactiae* simply does not colonise the teat skin (i.e. cannot be found in a viable form). Although this aspect has yet to be elucidated, the ambiguity in the results suggests that both situations may be true, but the implications are still to be established.

The PCR test was highly sensitive for detecting *Staph. aureus* and *Strep. agalactiae* in both milk and teat skin samples, whereas BC had a remarkably lower Se (RQ3 and RQ4, Manuscript II). When considering the association between teat skin colonisation and IMI, the positive PCR results from the teat skin should be considered as a risk of IMI, at least for *Strep. agalactiae*. When mastitis experts interpreted the diagnostic test patterns (Manuscript III), it appeared that positive PCR test results from milk were sometimes regarded as false positives – in principle decreasing the Se of PCR compared to the results from the LCA (Manuscript II). Positive BC test results were, however, taken more seriously (RQ7) by the experts. The diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae* were mainly consistently positive, thereby representing persistent infections according to the mastitis experts (RQ5 and RQ6). Such infections with consistently positive diagnostic test results would be easy to interpret and diagnose. However, some diagnostic test patterns were more inconsistent and were interpreted as dynamic infections or several different diagnoses were provided, which suggests that even with high Se and Sp of diagnostic tests, we should consider that there may be biological factors, courses of infection, and technical failures that make interpretation of test results challenging.

A more detailed discussion of the specific findings can be found in Manuscripts I-III. The findings can be used to improve recommendations for the diagnosis and control of *Staph. aureus* and *Strep. agalactiae* IMI. The study results will be discussed in the following sections, with a focus on the applicability in practice and in the context of previous findings in the literature.

5.1 Diagnosing *Staphylococcus aureus*

In line with previous studies (e.g. Costa et al., 2014; Haveri et al., 2008; Piccinini et al., 2009), we found that *Staph. aureus* colonised the teat skin (Manuscript I). Furthermore, *Staph. aureus* was detected on the teat skin using PCR. Previously, only BC has been used to assess the association between teat skin colonisation and IMI with *Staph. aureus* – an association we could confirm in AMS herds using BC, but not PCR. Based on PCR results, a high proportion of quarters were positive on teat skin without having an IMI (Table 4.2). The association between teat skin colonisation and IMI was therefore not confirmed (Manuscript I). However, the high number of teat skin samples found to be positive by PCR (that were not identified by BC) probably contributed to the high Se of PCR on teat skin in the LCA (Manuscript II). The question is whether these PCR-positive teat skin samples indicate a risk of IMI when no association is found and when we cannot confirm the presence of viable bacteria with PCR (Koskinen et al., 2009). To assess this further, a technique to distinguish between viable and non-viable bacteria should be applied, or the Se of BC should be increased to obtain isolates from the teat skin samples (as discussed in Manuscripts I and II). Isolates of viable bacterial cells from teat skin and milk from the same quarters should then be associated at molecular (ST) level, however, if causality should be obtained a different study design is needed, as milk could contaminate teat skin without being a risk of infection. Detection of *Staph. aureus* on teat skin using PCR is, however, important to consider when evaluating test results of non-aseptically collected samples, which would have a lower Sp compared to aseptically collected samples, as suggested by Mahmmod et al. (2013a).

Several sources of infection including cows skin, farm personnel and environmental reservoirs make it challenging to eradicate *Staph. aureus* from herds. As a result, this is often not the goal. Several STs can be found within a herd (Zadoks et al., 2011), and this was also demonstrated in the current study (Section 4.7). This supports a more environmental mode of transmission, even though *Staph. aureus* is considered to be a contagious udder pathogen. Nevertheless, control through hygiene and elimination of chronic IMI cases with high shedding of bacteria should be possible, but low cure rates should be kept in mind (Barkema et al., 2006; Rainard et al., 2017).

Multiple reservoirs of *Staph. aureus* increase the risk of obtaining a contaminated sample for diagnostic purposes; not only when collecting the milk samples on farm, but also when handling the sample in the laboratory. A relatively low cfu cut-off for BC, where only a single colony on the agar plate is needed to define IMI, further increases the risk of a sample being positive due to contamination. For the Mastit 4 PCR test, 100 cfu/mL would correspond to a Ct value of 34 (Katholm and Pedersen, 2016). The Ct value cut-off of 37 corresponds to 20 cfu/mL (Jørgen Katholm, DNA Diagnostic, personal communication), which means that the PCR test should be more sensitive than BC for detecting small amounts of bacteria, and thus also contaminants, which would generally be expected to occur in low concentrations. In principle, this would lower the Sp. However, according to the test evaluation in the LCA framework (Manuscript II), the PCR test should be preferable in terms of both Se and Sp for the diagnosis of *Staph. aureus*. The high Se estimates were in line with what was reported from previous studies estimating the test performance of BC and PCR for *Staph. aureus*, but in contrast to our findings, Sp estimates of BC and PCR assessed in an LCA framework were previously reported to be high for both BC and PCR (Mahmmod et al., 2013b; Nyman et al., 2016). The main differences between these and the current

study were that Mahmmod et al. (2013b) and Nyman et al. (2016) used non-aseptically collected milk samples for PCR and aseptically collected milk samples for BC, and evaluated test results at cow level. Furthermore, Mahmmod et al. (2013b) used a cut-off of 200 cfu/mL to indicate that BC samples were positive, which is likely to have increased the Sp of BC. Nevertheless, we in principle showed high Sp of BC due to the confirmation of all suspected *Staph. aureus* colonies by MALDI-TOF.

When test performance was evaluated based on the interpretation of 21-day diagnostic test patterns by researchers and mastitis experts, BC could be preferable in terms of Sp. The Se and Sp of both PCR and BC were high and comparable for the crude estimates and for researcher A and B (Figure 4.7). The Sp estimates for overall infection (based on the logistic regression model) were lower than the crude estimates, with the estimate for BC slightly higher than for PCR, indicating that some of the positive PCR results were considered false positives, in line with what can be seen in Figure 4.6. However, it should be noted that the estimates for overall infection were based on a random sample of the observations used in the crude estimates and as a result, the estimates are less certain (as also indicated by the larger CI in Figure 4.7).

The Se and Sp of SCC were generally lower than for BC and PCR. This is in line with the conclusion in previous studies assessing consecutive quarter-level milk samples. Walker et al. (2011) stated that duration and chronicity of an IMI with *Staph. aureus* was challenging to assess based on SCC, as they observed no relationship between SCC and other characteristics of the infections studied. Buelow et al. (1996) reported low Se (54 %) of SCC (cut-off 250,000 cells/mL) for composite milk samples, however, the same cut-off applied on quarter milk samples could be used for screening purposes as the obtained Se was 95%. Furthermore, SCC is not a part of the IMI definition according to IDF (IDF, 2011). As the experts were asked to diagnose IMI, it is therefore likely that SCC was not as important as BC and PCR in their interpretations. The positive test results of BC and PCR could, however, be contamination or teat canal colonisation (Hiitiö et al., 2016) instead of IMI, which is the "weak" point in the IMI definition. The experts' use of SCC for assigning a diagnosis may also be influenced by the pathogen detected. We presented the experts to diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae* which are considered major pathogens, yet the detection of minor pathogens as CNS could have caused different diagnoses as indicated in the research by Andersen et al. (2010). For minor pathogens, experts would probably, more or less deliberately, have included SCC in their diagnoses.

For *Staph. aureus,* it was possible to interpret the diagnosis and assign a code for 24 to 30 of the 30 possible expert diagnoses of the 21-day diagnostic test patterns (Table 4.4). This is higher than for *Strep. agalactiae,* perhaps because the *Staph. aureus* patterns were easier to describe and diagnose. This could be because mastitis experts are aware that *Staph. aureus* is able to hide intracellularly and be shed in cyclic patterns (Sears et al., 1990; Studer et al., 2008). The dynamic shedding patterns were therefore recognised (Figure 4.4), yet these patterns showed disagreement among the different tests, making it difficult to define this as a biological phenomenon, and more likely to be caused by test performance. Despite the previously reported cyclic shedding, we found that the majority of quarters investigated were consistently positive and mainly diagnosed as persistent infections. However, based on the plots (Figure 4.2), it

appears that there may be some false negative PCR test results that are not in line with the general perception that PCR should be more sensitive than BC.

5.2 Diagnosing *Streptococcus agalactiae*

Strep. agalactiae is considered an obligate parasite of the mammary gland and should be easily treatable (eliminated from the udder) with simple penicillin products (Keefe, 2012). In line with our findings regarding ST of isolates from two herds in the current study (Section 4.7), results from Danish (Mahmmod et al., 2015) and Norwegian (Jørgensen et al., 2016) dairy herds showed that a single ST dominated in each herd. This indicates that the mode of transmission is contagious (Zadoks et al., 2011) and eradication or control through antimicrobial treatment and simple "good milking management" should be achievable. Nevertheless, it appears that this is more challenging in practice, as the herd prevalence continues to increase nationally (Katholm et al., 2012). Possible oro-faecal transmission, as described by Jørgensen et al. (2016) could be an explanation for unsuccessful control and eradication when focusing on milking-time as the only risk of transmission. If an environmental reservoir of *Strep. agalactiae* exists, control of transmission must focus on general hygiene, as for example in the case of *Salmonella* Dublin control in cattle (Nielsen and Nielsen, 2012).

In Manuscript I, we concluded that *Strep. agalactiae* on teat skin detected by PCR increased the odds of IMI. A reservoir of *Strep. agalactiae* on teat skin might be the result of contact with milk from infected quarters during milking, or contact with an "environmental" reservoir during oro-faecal transmission. The low proportion of teat skin samples in which viable *Strep. agalactiae* was detected using BC (Manuscript I) indicates that it does not survive for a long time on teat skin. On the other hand, despite the ability of PCR to detect non-viable bacteria (Koskinen et al., 2009), it seems unlikely that all DNA detected by PCR on teat skin with Ct-values ranging from 24 to 37 were from dead bacteria. For milk samples, the BC detection limit of 100 cfu/mL corresponds to a Ct value of 28 (Katholm and Pedersen, 2016). The detection limit of 10 cfu/mL for teat skin samples (BC) would correspond to Ct 31 to 32 (Jørgen Katholm, DNA Diagnostic, personal communication), and samples with a Ct value below 32 should be detected by BC. There will be variation in test performance for milk and teat skin samples, but it is likely that BC has a low Se on teat skin samples as a result of competition with many other bacteria on the agar plate. Furthermore, the PCR test was not validated for teat skin samples before use in Manuscript II. Following discussion with the manufacturer, it was suggested that more cell material in the teat skin samples could increase the chance of spinning down bacteria in the DNA purification process, where there are two initial wash steps each followed by a spin of the cells and bacteria into a pellet. The Se of the PCR test may therefore increase if more cell material is present. This is probably in contrast to the performance of BC, where more cells (bacteria) are regarded as competitors to *Strep. agalactiae,* thereby potentially lowering the Se.

For quarter milk samples, the results from Manuscript II suggest that PCR should be the preferred test when a high Se is required. This is in line with the lower detection limit (Ct = 37 corresponds to less than 1 cfu/mL, Jørgen Katholm, DNA Diagnostic, personal communication). The Sp estimates were almost equal for PCR and BC. This is in line with other studies estimating the test performance of PCR and BC for *Strep. agalactiae* in an LCA framework (Table 2.1). In aseptically

collected milk samples, the risk of teat skin colonisation or contamination should be removed through preparation with 70% alcohol and as a result, the teat skin should not act as a reservoir or be a source of positive PCR results. When test performance was evaluated based on interpretations of 21-day diagnostic test patterns by researchers and mastitis experts, the Se of PCR was also estimated to be higher than that of BC, but BC had a higher Sp (Figure 4.7, crude estimates, researcher A and B), indicating that some of the positive PCR results were regarded false positives. As such, mastitis experts appear to be of the opinion that BC is still valuable in diagnosing IMI. In particular, a positive BC result for *Strep. agalactiae* should not be ignored. The SCC appeared to be a sensitive – but not very specific – tool for diagnosing *Strep. agalactiae*, which is in line with the perception that *Strep. agalactiae* IMI presents with high SCC (Keefe, 2012), yet there may be many other reasons for a high SCC. It therefore appears that experts would not rely on SCC for detecting IMI, which is also not appropriate according to the definition of IMI (IDF, 2011), but due to the high Se, SCC could be used as a tool for selecting cows for retesting. It should be noted that the Se of SCC for an overall infection (Figure 4.7) was remarkably low compared to the crude estimates and the estimates for the two researchers (A and B). This may be due to the selection of a random number of observations in the analysis described in Manuscript III. In addition, the Se of BC in this estimation was higher compared to the crude estimates and the estimates for researchers A and B.

It was possible to interpret and assign a code for 19 to 27 of the 30 possible expert diagnoses of each of the 21-day diagnostic test patterns (Table 4.5). This indicates that the interpretation of diagnostic test patterns was often not straightforward, or that the nomenclature of IMI and mastitis is inconsistent. Furthermore, the experts were limited to only four groups of diagnoses, whereas we needed nine codes for interpretation of the diagnoses. Nevertheless, more than half of the quarters were mainly diagnosed as persistently infected (Figure 4.3), the SCC was consistently above 100,000 cells/mL and few BC and PCR test results were negative. This fulfilled the definition of both IMI and mastitis. Two quarters were mainly diagnosed as not infected (Figure 4.6). The SCC was below 100,000 cells/mL and all BC and PCR test results were negative. However, these quarters were diagnosed by two experts as transient infections (Table 4.5), which could be due to the experts taking into consideration information about a positive screening result (day zero, not shown). If IMI is defined as detection of the pathogen in at least two out of three consecutive samples as suggested by Andersen et al. (2010), then both the quarters diagnosed as persistently infected and non-infected would be correctly identified. Furthermore, the chance of making a correct diagnosis based on a single sample would still be high in these consistent patterns. In contrast, the five quarters presented in Figure 4.5 would be more challenging to diagnose correctly, as the diagnosis was “undefined”. Whether they are biological or the result of low test accuracy, patterns like these would require more than three samplings or tests to rule out infection (e.g. for eradication purpose).

5.3 Factors that affect the choice of diagnostics

Several factors should be considered when choosing test strategies. A test strategy includes choices about sampling (automatic or manual), sample matrix (composite or quarter), preparation of the teats (aseptic or not), choice of diagnostic test (e.g. clinical signs, SCC, PCR, BC and combinations of these) and considerations about the decisions following the diagnosis (e.g. do

nothing, re-test, anti-microbial treatment, segregation, culling) and ultimately the impact on animal health and welfare, antimicrobial consumption and costs in addition to the farmer's motivation and goal. Some of the factors for consideration regarding the choice of test strategies are mentioned in the following section.

5.3.1 Factors that affect the diagnostic Se of BC and PCR on milk samples

Several factors affect the diagnostic Se of a test. Some of the most important factors affecting Se of BC and PCR are listed here:

- ***Analytical Se of the test*** where lower concentrations of bacteria can be identified (Koskinen et al., 2009) i.e. the Se of PCR is superior for both *Staph. aureus* and *Strep. agalactiae* (Manuscript I; Nyman et al., 2016; Holmøy et al., 2018).

- ***Cut-off values*** can be used to regulate the Se of the test (Andersen et al., 2010; Mahmmod et al., 2013b).

- ***Laboratory protocol and practices*** and considering the target pathogen in the test, i.e. primers for the target pathogen should be available in the PCR assay (Gillespie and Oliver, 2005) and special growth requirements and confirmatory tests should be considered in BC (NMC, 2004). Furthermore, the quality of laboratories and local herd veterinarians carrying out the tests may vary (Karlsmose et al., 2013).

- ***Number of samples testing positive*** where, for example, at least two positive samples out of three can be suggested as a strategy to increase Se (Andersen et al., 2010; Buelow et al., 1996; NMC, 2004).

- ***Sample matrix*** where the concentration of bacteria available in the sample is decreased in composite milk samples as a consequence of dilution of the milk from an infected quarter by milk from non-infected quarters – making quarter-level milk samples superior to composite milk samples.

5.3.2 Factors that affect the diagnostic Sp of BC and PCR on milk samples

In the same way as for Se, several factors will affect the diagnostic Sp of BC and PCR, and some of the most important are listed below:

- ***Analytical Sp of the test*** where PCR is superior to BC in principle, but may not be according to estimates from LCA. This could be due to a shift in the underlying condition identified by the LCA, where higher concentrations are favoured by BC and lower concentrations by PCR. In the data matrix underlying the LCA, the latent condition will be "somewhere in between" the outcome of the two tests, as driven by the data (and the prior information). This means that a theoretically superior Sp of the PCR can be outperformed by BC, leading to apparent false positives by the PCR.

- ***Teat skin reservoirs*** of *Staph. aureus* (Manuscript I; Haveri et al., 2008; Piccinini et al., 2009) and *Strep. agalactiae* (Manuscript I; Chodkowski, 1949) that have not been associated with IMI and may therefore be a result of contamination from the environment and milking equipment (Capurro et al., 2010; Jørgensen et al., 2016). If they are merely contaminants, they are in principle false positives. There is a risk that they can subsequently contribute to IMI, but if an IMI is not present then it should not be diagnosed unless precautions must be taken to avoid this IMI. Other reservoirs may be of similar importance.

- ***Carry-over*** from one cow to another during sampling in the milk recording scheme (Mahmmod et al., 2017, 2014). This is an effect of the less costly sampling strategy, where samples can be collected in a framework that is easier and cheaper for the farmer and herd veterinarian, but may result in false-positive samples when samples are not obtained aseptically. Furthermore, within herd prevalence may influence the risk of carry-over and contamination, i.e. if several cows are infected the risk of carry-over would probably increase. In general, false-positive test results could be minimised by increasing the test cut-off.

- ***Sample matrix*** where a positive quarter collected in a composite milk sample may result in a cow being classified with an IMI, irrespective of the other three quarters not being infected.

- ***Laboratory protocol and practices*** where BC in a non-accredited laboratory and not conducted by trained microbiologists increases the risk of contamination and erroneous readings, resulting in false positives due to e.g. a lack of confirmatory tests (Karlsmose et al., 2013).

5.3.3 Factors identified as important in the diagnosis

The above-mentioned factors can be relevant to decision makers when devising a test strategy. Furthermore, there may be a large variation in herd prevalence and management (Table 4.1), as well as differences in the ST of the pathogen (Table 4.8) that may need to be considered. Factors identified as being important considerations in the diagnosis include:

- Automatic sampling can be appealing because it is easy and sampling costs are low. It is appealing to many farmers based on annual testing of approximately 5,400,000 samples (RYK, 2016). The disadvantage is that aseptic procedures are compromised and samples are collected at cow level.

- Manual sampling allows for the collection of aseptic milk samples. This has the advantage of reducing the risk of contamination and combining sampling with an observation of inflammation signs in the same working procedure.

- Diagnoses based on quarter milk samples will be most accurate, as both Se and Sp are higher for quarter milk samples compared to composite milk samples, but sampling may be expensive.

- Clinical signs and SCC are the most appropriate measures for mastitis (IDF, 2011). These may, however, not be sufficient to treat legally in Denmark with products other than simple penicillin (including dry cow treatment).

- PCR is the test with the highest Se for diagnosing IMI.

- BC is the preferred test if high Sp of IMI is needed.

- SCC should be used as a screening tool and/or in combination with BC and PCR to assess IMI. Combining SCC and PCR on the same automatically collected composite milk sample in the milk control scheme may be appealing from a practical point of view.

- Segregation of infected animals in control or eradication programmes requires a high test Se to avoid the spread of pathogens.

- Antimicrobial treatment initiated to address any suffering of the animal requires a high test Se. To identify cows or quarters for antimicrobial treatment with the aim of decreasing the spread of pathogens within the herd, high test Sp is needed to avoid unnecessary use of antimicrobials.

- Culling of cows with chronic IMI can be cost-effective (Gussmann, 2018), but sampling costs, the cost of testing, and costs of false positives resulting in erroneous culling should be considered. High test Sp is needed if culling follows a positive test result.

- Doing nothing would be an option if the pathogen or test-pattern suggests it. Further studies are required for the two pathogens mentioned here, but some other pathogens are generally not treated (e.g. CNS).

5.4 Recommendations

This section presents my thoughts about test strategies and my recommendations for the interpretation of diagnostic test results based on results from the thesis and published literature.

Decisions should be made following a test result that indicates the treatment effect and cost-effectiveness. Therefore, the purpose of testing should be the first thing to include in a test strategy.

When diagnosing an IMI, it is important to remember that not all cows from which a pathogen has been identified will experience an IMI or an IMI that cannot resolve by itself. The ideal test can therefore distinguish between: a) non-IMI, b) IMI that can resolve without antimicrobials, c) IMI that require antimicrobials, and d) chronic IMI for which standard antimicrobial treatment has

limited effect – at least for *Staph. aureus*. This scenario will require minimal false positives from teat skin and environmental contamination, minimal false negatives, and minimal carry-over. It will also require repeated sampling to determine the nature of the infection and to increase the accuracy of the diagnosis. So far, the guidelines for repeated samples suggest that two out of three consecutive BC results should be positive for a quarter to be considered infected. Although such requirements may be well suited for research, they may not be suitable nor cost-effective in day-to-day on-farm mastitis diagnostics.

Automatic sampling via the milk recording scheme is less costly and time consuming than manual sampling, but sampling is only done monthly, at cow level and with non-aseptically collected samples. The milk recording scheme could be used as a screening tool, but all positive results should be retested to distinguish between: a) false positives; b) truly infected samples with persistent bacterial shedding and c) truly infected samples where the infection resolves. The risk of false positives caused by contamination would be of less concern if repeated testing were used. However, to reduce the number of samples per cow or quarter, retesting must be based on a sample that is obtained aseptically. A composite sample could be used to reduce the cost of testing. Furthermore, the SCC from the milk recording scheme has value in discriminating between infection with inflammation or mere carriage, but repeated SCC is required to determine the persistence of the infection. However, repeated PCR or BC on an aseptically collected sample may better address this purpose.

In conclusion, high Se is required to identify as many of the IMI as early as possible, but sampling must be logistically feasible. Using PCR for automatically collected milk recording samples seems to be an obvious choice, even though some information is lost compared to quarter milk samples. The results must be combined with SCC from the same recording in order to distinguish infection from contamination. Comparisons can be made to previous SCC from the same cow and used to increase the Sp of the SCC, or the SCC can be compared to expectations based on cows in the same parity and lactation (Græsbøll et al., 2016). Follow-up testing of PCR-positive samples can be achieved with both PCR and BC using aseptically collected samples. A negative result may require a third test to confirm whether any IMI has resolved – either by itself or following antimicrobial treatment – and to confirm that the cow or quarter is no longer a reservoir of pathogens that can spread within the herd.

5.4.1 Suggested interpretation of test results

For *Staph. aureus,* the risk of contamination and teat skin colonisation should be taken into account, especially when interpreting non-aseptically collected samples (Table 5.1). For *Strep. agalactiae,* the risk of false-positive test results is low, but false-negative test results are possible and it is recommended to combine tests or employ multiple sampling (Table 5.2). In general, the test results could be combined with information about the SCC. A quarter-level SCC above 100,000 cells/mL has the potential to be used for detecting IMI based on the results in Section 4.6, but the use of and cut-off for SCC at cow level is highly debated and therefore not directly included in these recommendations.

Table 5.1: Suggested interpretations of diagnostic test results for *Staphylococcus aureus* using different tests and samples. The recommendations are based on results from this thesis and published literature

Sample	Test	Result	Interpretation
Aseptically collected quarter milk sample	BC	Positive[1]	IMI is likely present, the risk of a false-positive sample is around 25%[a] as teat canal colonisation and contamination in the laboratory is also a risk
		Negative[2]	IMI could be present with 50%[b] risk of a false-negative; test with PCR and/or test a new sample 3 days later and combine information with results from SCC if available
	PCR	Positive[3]	IMI is likely present, the risk of a false-positive sample is < 4%*[a] but there may be contamination during the test procedure
		Negative[4]	IMI could still be present, the sample could be negative due to variation in shedding (false-negative), but the risk is low (< 20%[b]); combine information with results from SCC if available
Non-aseptically collected composite or quarter milk sample	BC	Positive[1]	IMI could be present, but the risk of a false-positive sample is likely higher than for BC on an aseptically collected sample (i.e. >25%) due to teat skin colonization and several other reservoirs of the bacteria; combine information with results from SCC if available
		Negative[2]	IMI could be present, the risk of a false-negative is as likely as for aseptically collected samples (50%); repeat testing with PCR on an aseptically collected sample and combine information with results from SCC if available
	PCR	Positive[3]	IMI could be present, but the risk of a false-positive sample is likely higher than for aseptically collected samples (maybe 10 to 40%*) due to teat skin colonisation and several other reservoirs of the bacteria
		Negative[4]	IMI can still be present, the sample could be negative due to variation in shedding (false-negative), but the risk is as low as for aseptically collected samples (< 20%); combine information with results from SCC if available

[1] BC is considered positive at ≥ 100 cfu/mL; [2] BC is considered negative if no growth is assessed using at least 0.01 mL of milk for plating; [3] PCR is considered positive at Ct ≤ 37; [4] PCR is considered negative at Ct > 37 [a] The risk of false-positives is based on estimates from Manuscript II and calculated as 100% – Specificity from informative estimates; [b] The risk of false negatives is based on estimates from Manuscript II and calculated as 100% – Sensitivity from informative estimates; * Based on the expert analysis in Manuscript III, the risk of false-positives was most likely around 40%.

Table 5.2: Suggested interpretations of diagnostic test results for *Streptococcus agalactiae* using different tests and samples. The recommendations are based on results from this thesis and published literature

Sample	Test	Result	Interpretation
Aseptically collected quarter milk sample	BC	Positive[1]	IMI is present, the risk of a false-positive sample is 1%[a]
		Negative[2]	IMI could be present with 60%[b] risk of a false-negative; test with PCR and/or test a new sample 3 days later and combine information with results from SCC if available
	PCR	Positive[3]	IMI is present, the risk of a false-positive sample is < 3% [a]
		Negative[4]	IMI could still be present, the sample could be negative due to variation in shedding, but the risk is low (< 12% [b]); combine information with results from SCC if available
Non-aseptically collected composite or quarter milk sample	BC	Positive[1]	IMI is present, the risk of a false-positive sample is as likely as for aseptically collected samples (1%), as teat skin colonisation is unlikely detected by BC in non-IMI quarters
		Negative[2]	IMI could be present with the same or higher risk of false-negative as for an aseptically collected sample; repeat testing with PCR on aseptically collected sample and combine information with results from SCC if available
	PCR	Positive[3]	IMI most likely present, but this could also be bacteria from teat skin or environment and the risk of a false-positive sample will be higher than 3% due to the non-aseptically collection
		Negative[4]	IMI could still be present, the sample could be negative due to variation in shedding, but the risk is as low as for an aseptically collected sample (< 12%); combine information with results from SCC if available

[1] Bacterial culture (BC) is considered positive at ≥ 100 cfu/mL; [2] BC is considered negative if no growth is assessed using at least 0.01 mL of milk for plating; [3] PCR is considered positive at Ct ≤ 37; [4] PCR is considered negative at Ct > 37; [a] The risk of false-positives is based on estimates from Manuscript II and calculated as 100% – Specificity from informative estimates; [b] The risk of false negatives is based on estimates from Manuscript II and calculated as 100% – Sensitivity from informative estimates

5.5 Perspectives

The results of this thesis have contributed to a better understanding of diagnostic test results of *Staph. aureus* and *Strep. agalactiae*, and have been used to suggest some recommendations in Section 5.4. The recommendations can be used and expanded in future as more knowledge is gained on the topic. Furthermore, the recommendations should be adapted to the changing needs and regulations of different countries, as well as the aim of use within herds.

Understanding transmission and the importance of reservoirs in terms of controlling IMI with *Staph. aureus* and *Strep. agalactiae* provides various prospects for future studies. To identify causality in relation to the association of pathogens present on teat skin and IMI (as discussed in Manuscript I), a longitudinal study could be carried out. Furthermore, the association should be evaluated at ST level, and it would be interesting to assess the ST of the pathogens in relation to herd characteristics and management.

To further improve the interpretation and use of the PCR test, it would be highly relevant to distinguish between the viable and non-viable bacteria detected. Furthermore, it would be relevant to assess how we can differentiate between infection, colonisation and contamination in relation to diagnostics.

As mentioned in the discussion of Manuscript III, the expert analysis could be repeated with the developed codes given, in order to validate the identified IMI diagnoses. Furthermore, experts could provide information for the suggested interpretation of these diagnoses.

In general, test strategies should be implemented and evaluated at farm level, and a cost-benefit analysis should be considered in order to assess the relevance of different strategies.

6 Conclusions

This PhD project investigated diagnostic test performance, infection dynamics, and the teat skin as a reservoir of the contagious udder pathogens *Staph. aureus* and *Strep. agalactiae*, with the aim to improve diagnostics of *Staph. aureus* and *Strep. agalactiae*. The results showed that precautions are needed when diagnosing IMI with *Staph. aureus* and *Strep. agalactiae*.

Based on the research questions in section 1.1, the following conclusions can be reached.

RQ1: The odds of *Staph. aureus* IMI were high (7.8 [2.9; 20.6] times higher) if *Staph. aureus* was detected by BC on the teat skin of the same quarter. PCR testing found no association between the detection of *Staph. aureus* on teat skin and in milk of the same quarter, probably due to the PCR test detecting lower concentrations or inactivated bacteria on the teat skin of quarters with no IMI.

RQ2: The proportion of quarters for which *Strep. agalactiae* was detected on teat skin with BC was low (0.35%), either because BC of *Strep. agalactiae* is insufficient for non-milk samples, or because *Strep. agalactiae* does not colonise teat skin. However, the number of positive samples was too low to carry out statistical analysis. For *Strep. agalactiae* detected by PCR, the odds of *Strep. agalactiae* IMI were 3.8 [1.4; 10.1] times higher if it was detected on teat skin compared to a quarter with a negative teat skin sample.

RQ3: The PCR test was the most sensitive and specific of the tests in the detection of *Staph. aureus*. The Se and Sp of PCR for detecting *Staph. aureus* in milk samples were 0.95 [0.82; 1.00] and 0.99 [0.97; 1.00], respectively, whereas the Se and Sp of BC were 0.54 [0.46; 0.62] and 0.77 [0.73; 0.81], respectively. The Se and Sp of PCR for detecting *Staph. aureus* in teat skin samples were 0.94 [0.80; 0.99] and 0.98 [0.94; 1.00], respectively, while the Se and Sp of BC were 0.44 [0.36; 0.52] and 0.74 [0.70; 0.78], respectively.

RQ4: The PCR test was the most sensitive in the detection of *Strep. agalactiae*, whereas BC and PCR had similar Sp. The Se and Sp of PCR for detecting *Strep. agalactiae* in milk were 0.97 [0.88; 1.00] and 0.99 [0.97; 1.00] , respectively, whereas the Se and Sp of BC were 0.41 [0.35; 0.47] and 1.00 [0.99; 1.00], respectively. The Se and Sp of PCR for detecting *Strep. agalactiae* in teat skin samples were estimated to be 0.97 [0.87; 1.00] and 0.96 [0.89; 1.00], respectively, while the Se and Sp of BC were 0.33 [0.27; 0.41] and 1.00 [0.99; 1.00], respectively.

RQ5: The short-term (21 day) diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae* are mainly consistent. However, there was variation in the shedding patterns. In addition, non-infected quarters may be detected if quarters are included based on a single positive PCR sample.

RQ6: Based on short-term diagnostic test patterns, mastitis experts identified mainly persistent infections, including persistent infections with false-negative test results. Furthermore, dynamic infections, new infections, new infections with false-negative test results, transient infections, resolving infections and healthy quarters with and without false-positive test results were identified.

RQ7: The PCR and BC test performance was generally high when different infection types were defined by mastitis experts. However, the Sp for detecting an overall infection with *Staph. aureus* was low, and the same was true of the Sp for detecting new infections with *Strep. agalactiae*. The test performance of SCC (with a cut-off at 100,000 cells/mL) for diagnosing IMI with *Staph. aureus* or *Strep. agalactiae* was generally lower than for PCR and BC. Major variation in the Se and Sp estimates was observed when dealing with new infections and transient infections, perhaps because mastitis experts were in disagreement, and consistency in terminology and application of mastitis diagnoses could be investigated further.

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8 Manuscripts

Manuscript I

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Manuskript II

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Manuscript I

Association between teat skin colonization and intramammary infection with *Staphylococcus aureus* and *Streptococcus agalactiae* in herds with automatic milking systems

Line Svennesen,[1*] Søren S. Nielsen,[1] Yasser S. Mahmmod,[1,2†] Volker Krömker,[3] Karl Pedersen,[4] and Ilka C. Klaas[1‡]

[1]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
[2]Infectious Diseases, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia Province, Egypt
[3]Department of Microbiology, University of Applied Sciences and Arts, 30453 Hannover, Germany
[4]National Veterinary Institute, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
[†]Current address: IRTA, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona, 08193-Bellaterra, Barcelona, Spain.
[‡]Current address: DeLaval International AB, Tumba, Sweden 14741.

*Corresponding author: Line Svennesen, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegårdsvej 2, DK-1870 Frederiksberg C, Denmark. Email: line.svennesen@sund.ku.dk. Phone: +45-35331987

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Abstract

The objective of this study was to investigate the association between teat skin colonization and intramammary infection (IMI) with *Staphylococcus aureus* or *Streptococcus agalactiae* at the quarter level in herds with automatic milking systems. Milk and teat skin samples from 1,142 quarters were collected from 300 cows with somatic cell count >200,000 cells/mL from 8 herds positive for *Strep. agalactiae*. All milk and teat skin samples were cultured on calf blood agar and selective media. A subset of samples from 287 quarters was further analyzed using a PCR assay (Mastit4 PCR; DNA Diagnostic A/S, Risskov, Denmark). Bacterial culture detected *Staph. aureus* in 93 (8.1%) of the milk samples and 75 (6.6%) of the teat skin samples. Of these, 15 (1.3%) quarters were positive in both the teat skin and milk samples. *Streptococcus agalactiae* was cultured in 84 (7.4%) of the milk samples and 4 (0.35%) of the teat skin samples. Of these, 3 (0.26%) quarters were positive in both the teat skin and milk samples. The PCR detected *Staph. aureus* in 29 (10%) of the milk samples and 45 (16%) of the teat skin samples. Of these, 2 (0.7%) quarters were positive in both the teat skin and milk samples. *Streptococcus agalactiae* was detected in 40 (14%) of the milk samples and 51 (18%) of the teat skin samples. Of these, 16 (5.6%) quarters were positive in both the teat skin and milk samples. Logistic regression was used to investigate the association between teat skin colonization and IMI at the quarter level. Based on bacterial culture results, teat skin colonization with *Staph. aureus* resulted in 7.8 (95% confidence interval: 2.9; 20.6) times higher odds of *Staph. aureus* IMI, whereas herd was observed as a major confounder. However, results from the PCR analyses did not support this association. *Streptococcus agalactiae* was isolated from the teat skin with both PCR and bacterial culture, but the number of positive teat skin samples detected by culture was too low to proceed with further analysis. Based on the PCR results, *Strep. agalactiae* on teat skin resulted in 3.8 (1.4; 10.1) times higher odds of *Strep. agalactiae* IMI. Our results suggest that *Staph. aureus* and *Strep. agalactiae* on teat skin may be a risk factor for IMI with the same pathogens. Focus on proper teat skin hygiene is therefore recommended also in AMS.

Key words: B-streptococci, contagious mastitis, dairy cattle, PCR

Introduction

Intramammary infections with *Staphylococcus aureus* and *Streptococcus agalactiae* are usually associated with subclinical infections that reduce milk quality and production (Keefe, 2012). *Staphylococcus aureus* and *Strep. agalactiae* are traditionally considered contagious mastitis pathogens that transfer from cow to cow during milking [e.g., by contaminated milking equipment and milkers' hands (Keefe, 2012)]. The teat skin might therefore serve as a reservoir of pathogens that enter the udder through the teat canal and cause IMI.

It is generally agreed that *Staph. aureus* can be isolated from the teat skin and other extramammary body sites (Larsen et al., 2000; Haveri et al., 2008; da Costa et al., 2014), and teat skin colonization with *Staph. aureus* has subsequently been epidemiologically associated with *Staph. aureus* IMI in heifers and cows (Roberson et al., 1994; da Costa et al., 2014). Furthermore, the same pulsed-field gel electrophoresis types of *Staph. aureus* have been isolated from both teat skin and milk within herd, cow, or quarter (Haveri et al., 2008; Piccinini et al., 2009; da Costa et al., 2014). To the best of our knowledge, the role of the teat skin as a source of *Staph. aureus* IMI has not been investigated in herds with automatic milking systems (AMS), where milking hygiene and teat cleaning may differ from conventional milking systems (Hovinen and Pyörälä, 2011). Lactating dairy cows can be milked several times a day in AMS without being in contact with human hands, and up to 60 cows can be milked with the same milking unit (Rodenburg, 2017). These factors are likely to affect teat skin colonization and the transmission of contagious mastitis pathogens.

To our knowledge, the association between *Strep. agalactiae* on teat skin and in milk has not yet been studied. *Streptococcus agalactiae* was isolated from teat skin and other areas on cows and in cowsheds by Chodkowski (1949)). However, *Strep. agalactiae* was still considered an obligate intramammary pathogen (Keefe, 1997) until an environmental reservoir was recently suggested by Jørgensen et al. (2016)), as *Strep. agalactiae* was isolated from, for example, water troughs, milking robots, and stalls, and the rectum and vagina of the cows (Jørgensen et al., 2016; Farre et al., 2017; Henriksen et al., 2017).

Although bacterial culture has mostly been used to study the above-mentioned aspects, PCR has generally been used more frequently in recent years, particularly in European countries (Koskinen et al., 2010; Mahmmod et al., 2013a,b; Nyman et al., 2016). The PCR assay may have a higher analytical sensitivity, and the potential to detect a broader range of bacteria simultaneously without additional diagnostic efforts. However, the PCR assay may detect nonviable bacteria (Koskinen et al., 2009), which have no important role in transmission. Nonviable bacteria may be considered as false-positive reactions in this regard, whereas viable bacteria only are detected by bacterial culture. However, nonviable bacteria may also be an expression of past exposure, where the bacteria have been killed by teat disinfectants or other circumstances. As such, bacterial culture may be considered more specific for some interpretations. Therefore, the use of both methods may provide information on slightly different aspects of the pathogens in the udder and the surroundings.

Knowledge of pathogen reservoirs is crucial in the management of *Staph. aureus* and *Strep. agalactiae* transmission to prevent IMI. In large dairy herds and herds with AMS, controlling transmission related to milking is fundamental in reducing the number of new infections with contagious mastitis pathogens. The objective of the current study was to investigate the association between colonization of the teat skin and IMI with *Staph. aureus* or *Strep. agalactiae* in the same quarter in dairy herds with AMS. The results provide new knowledge to improve strategies for the control of *Staph. aureus* and *Strep. agalactiae* in dairy herds.

Materials and Methods

Herds and Animals

Eight Danish dairy herds were selected for inclusion in this field study by convenience sampling, and were visited once between February and May 2017. The herd-level inclusion criteria were as follows: at least 3 automatic milking units, a positive *Strep. agalactiae* status, and a willingness from the farmer to participate. The status of *Strep. agalactiae* was based on the annual screening of bulk tank milk samples (BTMS) in 2016 using the quantitative PCR Mastit4 test (DNA Diagnostic A/S, Risskov, Denmark). To confirm that the herds were still positive for *Strep. agalactiae*, another 3 BTMS from each herd were tested with Mastit4 in January 2017. The herds were considered positive if at least 2 out of 3 BTMS were positive for *Strep. agalactiae* with a PCR cycle threshold (Ct) ≤ 32.

Between 30 and 40 cows with SCC >200,000 cells/mL at the last milk recording were randomly selected from each herd using a random number generator in SAS 9.4 (SAS Institute Inc., Cary, NC). Cows that received antimicrobial treatments during the 4 wk before sample collection or that were dried off in the 5 to 33 d between the last milk recording and sampling were excluded. All functional quarters of the selected cows were sampled. Herd and sample size details are presented in Table 1.

Table 1. Herd characteristics (herd size, number of automatic milking units (AMU), milk production, geometric bulk tank SCC and breed) at the time of re-testing bulk tank milk samples; post-milking teat disinfection used at the time of sampling (teat spray in milking robots); Cycle threshold (Ct) values of *Strep. agalactiae* and *Staph. aureus* in bulk tank milk samples (from the three re-tests), and number of cows with high SCC (> 200,000 cells/mL) at last milk recording before sampling - from which the number of sampled cows and quarters were selected

Herd	Herd size[1]	No. of AMU	Milk production[2]	SCC[3]	Breed[4]	Post-milking teat disinfection	Ct value *Strep. agalactiae* bulk milk	Ct value *Staph. aureus* bulk milk	No. of cows with high SCC[5]	No. of cows sampled (%)[6]
H1	267	4	10,973	183	100	0.3% iodine	30, 40, 23	32, 29, 30	43	29 (67)
H2	198	3	11,098	211	91	0.3% iodine	25, 32, 22	25, 40, 31	43	37 (86)
H3	344	7	10,733	216	93	1% lactic acid	29, 26, 25	40, 33, 40	74	39 (53)
H4	298	5	11,412	199	83	0.75% iodine	25, 23, 21	40, 21, 28	60	38 (63)
H5	218	4	9,024	255	63	0.3% iodine	26, 24, 20	32, 29, 28	49	39 (80)
H6	247	4	11,701	252	100	0.3% iodine	25, 28, 40	35, 26, 40	59	40 (68)
H7	333	6	11,909	192	98	0.15% iodine	24, 25, 24	25, 24, 29	50	38 (76)
H8	244	4	11,020	338	98	0.15% iodine	24, 21, 22	31, 28, 27	79	40 (51)

[1]Includes both lactating and dry cows. [2]Estimated kg ECM/cow per year. [3]× 1,000 cells/mL: Geometric mean of bulk tank SCC within last 3 mo. [4]Danish Holstein (%). [5]SCC > 200,000 cells/mL at last milk recording. [6]% of cows with somatic cell count > 200,000 cells/mL.

Collection of Milk and Teat Skin Samples

Cows were restrained in headlocks during sampling. Teats were cleaned with dry paper towels until visually clean, with at least one piece of paper used for each quarter. The teat skin samples were collected using a modified wet-and-dry swab technique (Paduch and Kroemker, 2011). Briefly, a sterile rayon swab (DaklaPack, Glostrup, Denmark) was moistened with 1/4 Ringer's solution (Merck, Darmstadt, Germany) and rotated 360° around the teat at a distance of 1 cm from the teat canal orifice. The same procedure was followed using a dry swab and the tips of both swabs were transferred into the same tube containing 2 mL of 1/4 Ringer's solution. Data on Danish Holstein cows suggest that the mean teat diameter is 2.3 cm (unpublished genetic evaluation data, SEGES P/S, Aarhus, Denmark). We therefore estimated the sampled area of the teat to be 7.2 cm2 based on a swab length of 1 cm.

Teat end hyperkeratosis was scored using a 4-point scale (Mein et al., 2001) immediately after the teat skin samples were collected. The teats were forestripped and milk samples were collected aseptically following the procedures described by the National Mastitis Council (Hogan et al., 1999) as follows: the teat end was disinfected with cotton pads moistened in 70% ethanol, and 2 to 3 squirts of milk were discarded before 5 to 10 mL was collected in a sterile tube.

Milk and teat skin samples were stored at 5°C before laboratory analysis on the following day.

Teat skin sampling and hyperkeratosis scoring was carried out by the same 3 trained veterinarians. Milk samples were collected by 3 milk quality technicians. A veterinarian and a milk quality technician worked together as a sampling team, and the sampling team and sequence were recorded. Disposable gloves were worn when collecting samples, and these were replaced between teat skin and milk sampling, and between each animal. The sampling time was on average 10 min/cow per team.

Laboratory Procedures

Teat skin samples were acclimatized and vortexed for 20 s before the swab tips were removed with a pair of sterile tweezers. A whole agar plate was inoculated with 100 µL of the swab solution. Milk samples were acclimatized and vortexed for 10 s on a vortex mixer and 10 µL of milk was streaked onto a quadrant of an agar plate using a sterile disposable loop.

All samples were plated on blood agar (5% sheep blood), chromogenic agar selective for staphylococci (SaSelect, Bio-Rad, Hercules, CA), and modified Edwards medium [Oxoid, Roskilde, Denmark, supplemented with 5% calf blood and 2% filtrate of a β-toxin-producing *Staph. aureus* prepared as described by Jørgensen et al. (2016))]. Plates were incubated aerobically at 37°C and examined for growth at 24 and 48 h.

Colonies of *Staph. aureus* and *Strep. agalactiae* were phenotypically identified based on colony morphology and hemolysis on blood agar. *Staphylococcus aureus* was identified as pink colonies on SaSelect agar, as per the manufacturer's instructions. On the modified Edwards medium, *Strep. agalactiae* was identified as esculin-negative, blue to colorless colonies with a CAMP (Christie–Atkins–Munch–Petersen) reaction. Colony counts were recorded for each plate. A single colony was sub-cultured from each suspected *Staph. aureus* or *Strep. agalactiae* colony type on a new blood agar and incubated for 24 h to be freshly submitted to MALDI-TOF (Bruker Biotyper software system, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany), as previously described (Mahmmod et al., 2018). The identification of *Staph. aureus* was confirmed with MALDI-TOF, whereas *Strep. agalactiae* was confirmed with a slide agglutination test for Lancefield group B (PathoDxtra Strep Grouping Kit, Thermo Fisher Scientific, Waltham, MA) or MALDI-TOF. The finding of CNS (also confirmed by MALDI-TOF) on SaSelect agar was registered for both milk and teat skin samples, and “other growth” was registered only for milk samples, including environmental streptococci, Enterococcus, and Aerococcus spp. If more than 2 dominant colony types were present, the agar plate was not considered in the reading for milk samples. For teat skin samples, contamination was not considered due to the contaminated origin of the sample.

Teat skin and milk samples from right rear quarters were further analyzed with the Mastit4 PCR test. A FLOQswab (COPAN ITALIA spa, Brescia, Italy) was immersed in the original samples immediately after streaking for bacterial culture. Swabs were shipped on the same day to the analysis laboratory of DNA Diagnostic A/S for analysis 1 or 2 d later.

Statistical Analysis

Two data sets were created: one based on culture and the other on PCR. Information on parity, DIM, and SCC on the most recent milk recording day was extracted from the Danish Cattle Database (SEGES P/S, Aarhus, Denmark). The time between milking and sampling was calculated based on an estimated sampling time and the time of milking from the AMS. The estimated sampling time was calculated by adding 10 min per cow to the first sampling time in each herd, and using the sampling sequence within each sampling team. Somatic cell count was included as an indicator of infection, and hyperkeratosis has been associated with some pathogens (Guarín et al., 2017), whereas time from milking was hypothesized to affect the risk of contamination from the environment and effect of postmilking teat disinfection.

Culture results were dichotomized, and a milk sample with ≥1 cfu/10 μL (100 cfu/mL) on either blood agar or the selective agar was considered to be positive. Teat skin samples were considered positive with ≥1 cfu/100 μL (10 cfu/mL). For the PCR test, Ct values ≤37 were considered positive, as suggested by the manufacturer.

The dichotomous variables of *Staph. aureus* or *Strep. agalactiae* in milk (IMI) detected by culture or PCR were used as outcome variables for separate models. The explanatory variable of primary interest was teat skin colonization with the same pathogen and test as the outcome. Parity, DIM, SCC at last milk recording, time since last milking, hyperkeratosis score, and various other pathogens detected in milk and teat skin samples were all included as categorical variables (Table 2) to adjust for different cow-, milk-, and teat-related effects. The categories were created so the numbers of observations in each category were as close to each other as possible while still being meaningful.

Cross-tabulation of all explanatory variables with the 2 outcome variables was performed for both data sets. A logistic regression model was used to estimate the association between teat skin colonization and IMI with the same mastitis pathogen. All variables were first assessed in univariable models, and those with a P-value <0.20 from an F-test were offered to a multivariable model. The full model was reduced using backward elimination based on Hannan-Quinn information criterion, which was used to assess model fit along with model convergence. Furthermore, the Pearson $\chi 2$-statistic was used to determine whether unexplained extra-binomial variation was present. For the culture data, the cow identifier was included as a random effect to control for similarity between quarters nested within cows. Herd could not be included as a random effect due to a lack of convergence. Herd was included as a random effect in the PCR data with only one observation per cow to control for cows nested within herds, using a simple diagonal covariance structure. In random effect models, subject specific odds ratios were reported. The analyses were carried out using the Glimmix procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC).

Table 2. Cross-tabulation of *Staphylococcus aureus* and *Streptococcus agalactiae* culture positivity in milk samples from 1,142 quarters and the distribution across different variables and *P*-values from univariable statistics (the overall prevalence and herd prevalence are shown at the quarter level)

			Staph. aureus in milk[1]			*Strep. agalactiae* in milk[1,5]	
Variable	Level	N	# positive	% positive	P-value univariable statistics	# positive	% positive
Overall		1,142	93	8.1		84	7.4
Staph. aureus on teat skin[1]	Negative	1,067	78	7.3	0.012	71	6.7
	Positive	75	15	20.0		13	17.3
Strep. agalactiae on teat skin[1]	Negative	1,138	93	8.2	NA[2]	81	7.1
	Positive	4	0	0.0		3	75.0
Herd	H1	111	18	16.2	< 0.0001	1	0.9
	H2	145	4	2.8		0	0.0
	H3	148	3	2.0		10	6.8
	H4	144	3	2.1		10	6.9
	H5	149	3	2.0		17	11.4
	H6	154	2	1.3		3	2.0
	H7	141	8	5.7		29	20.6
	H8	150	52	34.7		14	9.3
Hyperkeratosis	1 (no ring)	64	3	4.7	0.022	3	4.7
	2 (smooth ring)	777	48	6.2		43	5.5
	3 (rough ring)	227	27	11.9		21	9.3
	4 (very rough)	74	15	20.3		17	23.0
Parity	1	221	21	9.5	0.88	6	2.7
	2	378	30	8.0		30	8.0
	3+	543	42	7.7		48	8.8
DIM	< 90	291	10	3.4	0.075	10	3.4
	90 – 199	345	26	7.5		20	5.8
	≥ 200	506	57	11.3		54	10.7
SCC (× 1,000 cells/mL)[3]	200 – 399	467	39	8.4	0.9	14	3.0
	400 – 999	383	27	7.1		44	11.5
	≥ 1,000	292	27	9.3		26	8.9
Time since last milking[4]	± 0.5 h[5]	87	0	0.0	0.35	2	2.3
	0.5 h to 2.5 h	265	16	6.0		7	2.6
	2.5 h to 5 h	264	27	10.2		9	3.4
	5 h to 9 h	269	26	9.7		26	9.7
	≥ 9 h	257	24	9.3		40	15.6
CNS teat skin[1]	Negative	26	4	15.4	0.29	1	3.9
	Positive	1,116	89	8.0		83	7.4
CNS milk[1]	Negative	500	47	9.4	0.043	27	5.4
	Positive	642	46	7.2		57	8.9
Staph. aureus in milk[1]	Negative	1,049	NA	NA	NA	77	7.3
	Positive	93	NA	NA		7	7.5
Strep. agalactiae in milk[1]	Negative	1,058	86	8.1	0.52	NA	NA
	Positive	84	7	8.3		NA	NA
Other growth in milk[6]	Negative	753	73	9.7	0.0003	71	9.4
	Positive	389	20	5.1		13	3.3

[1]Culture positive: ≥ 1 colony on the agar plate, corresponding to ≥ 100 cfu/mL milk and ≥ 10 cfu/mL teat skin sample. [2] NA = not applicable, too few data to do further analysis on *Strep. agalactiae* thus no univariable statistics to be shown. [3] SCC from last milk recording 5 to 33 d before sampling. [4] Time since last milking, calculated based on estimated sampling time and milking time from AMS. [5] Time category "± 0.5 h" merged with "0.5 h to 2.5 h" in univariable statistics due to zero positive *Staph. aureus* quarters (outcome variable) in time category "± 0.5 h". [6] Growth other than CNS, *Staph. aureus* and *Strep. agalactiae*

Results

Culture Results

Milk and teat skin samples were cultured from 1,142 quarters from 300 cows. *Staphylococcus aureus* was detected in 93 (8.1%) of the milk samples and 75 (6.6%) of the teat skin samples. Of these, 15 (1.3%) quarters were positive in both the teat skin and milk samples. *Streptococcus agalactiae* was detected in 84 (7.4%) of the milk samples and 4 (0.35%) of the teat skin samples. Of these, 3 (0.26%) quarters were positive in both the teat skin and milk samples, and these 3 quarters were from the same cow. Only one quarter was teat skin positive and milk negative. This was a quarter from a cow where *Strep. agalactiae* was isolated from milk in 2 other quarters.

The distribution of positive milk samples for *Staph. aureus* and *Strep. agalactiae* detected by culture is shown in Table 2. Due to the low number of positive samples for *Strep. agalactiae* in teat skin, logistic regression was only performed for *Staph. aureus*. Furthermore, *Strep. agalactiae* on teat skin was not offered the *Staph. aureus* model as a potential explanatory variable. Results from the univariable statistics are shown in Table 2, with herd, hyperkeratosis score, DIM, CNS, and other growth in milk offered to the multivariable model along with teat skin colonization with *Staph. aureus*. In the final multivariable analysis, the odds of *Staph. aureus* IMI were 7.8 [95% CI; 2.9–20.6] times higher in a quarter where *Staph. aureus* was isolated from teat skin compared with a quarter with a *Staph. aureus*-negative teat skin sample (Table 3). The odds also increased with increasing hyperkeratosis score, whereas the presence of other pathogens in the milk had a negative effect on the odds of *Staph. aureus* IMI. *Staphylococcus aureus* on teat skin and "other pathogens" in milk were confounded by herd, whereas the unadjusted odds ratio was 3.2 (based on data in Table 2) and the adjusted odds ratio was 7.8 (Table 3), and this change was primarily due to the addition of herd to the model.

Table 3. Results from the multivariable logistic regression model of association between *Staphylococcus aureus* culture positivity in quarter milk and teat skin samples from 1,142 quarters

Variable	Level	Estimate	SE	OR	95% CI		P-value
Staph. aureus on teat	Negative	0		Referemt			
skin	Positive	2.05	0.5	7.76	2.92	20.6	<0.0001
Herd	H1	0		Referent			
	H2	-2.07	0.64	0.13	0.036	0.45	0.0013
	H3	-3.06	0.74	0.047	0.011	0.2	<0.0001
	H4	-2.69	0.73	0.068	0.016	0.29	0.0002
	H5	-3.1	0.75	0.045	0.01	0.2	<0.0001
	H6	-3.33	0.83	0.036	0.007	0.18	<0.0001
	H7	-2.68	0.63	0.069	0.02	0.24	<0.0001
	H8	0.91	0.42	2.47	1.08	5.69	0.033
Hyperkeratosis	1 (no ring)	0		Referent			
	2 (smooth ring)	0.1	0.71	1.11	0.27	4.49	0.89
	3 (rough ring)	0.93	0.75	2.53	0.58	11.0	0.22
	4 (very rough)	2.19	0.85	8.91	1.69	46.8	0.0099
Other growth in milk	Negative	0		Referent			
	Positive	-1.25	0.36	0.29	0.14	0.58	0.0005

PCR Results

Milk and teat skin samples from 287 right rear quarters were analyzed with PCR. *Staphylococcus aureus* was detected in 29 (10%) of the milk samples and 45 (16%) of the teat skin samples. Of these, 2 (0.7%) quarters were positive in both the teat skin and milk samples. *Streptococcus agalactiae* was detected in 40 (14%) of the milk samples and 51 (18%) of the teat skin samples. Of these, 16 (5.6%) quarters were positive in both the teat skin and milk samples.

The range of Ct values for *Staph. aureus* in samples considered positive was (13–37) in milk and (27–37) in teat skin. For *Strep. agalactiae*, the Ct ranges were (10–37) and (24–37) in milk and teat skin samples, respectively. Three teat skin samples had Ct values between 37 and 40 for *Staph. aureus* and were thus above the chosen cut-off.

The distributions of positive milk samples for *Staph. aureus* and *Strep. agalactiae* detected by PCR are shown in Table 4. With only one quarter per cow, the distribution of parity, DIM, and SCC represents the sampled population at cow level. Results from the univariable statistics for *Staph. aureus* IMI are shown in Table 4. Hyperkeratosis, parity, DIM, and CNS on teat skin met the criteria for inclusion in the multivariable analysis ($P < 0.20$). The explanatory variable of primary interest (*Staph. aureus* on teat skin), did not meet the criteria, and as a consequence, no multivariable analysis was done on *Staph. aureus* IMI detected by PCR.

Results from the univariable statistics for *Strep. agalactiae* IMI are shown in Table 4, with the following variables offered to the multivariable model: *Strep. agalactiae* on teat skin, hyperkeratosis, parity, SCC, and CNS on teat skin.

In the final multivariable analysis, the odds of *Strep. agalactiae* IMI were 3.8 (95% CI; 1.4–10.1) times higher in a quarter that was *Strep. agalactiae*-positive on teat skin compared with a quarter with a teat skin sample negative for *Strep. agalactiae* (Table 5). The odds of a quarter having *Strep. agalactiae* in milk were also higher with increasing parity or when PCR showed the teat skin to be positive for CNS. Hyperkeratosis was confounded by SCC at last milk recording [e.g., the odds ratio for hyperkeratosis score 4 compared with scores 1 and 2 was 2.0 (based on the data in Table 4), whereas it was 3.5 in the multivariable model (Table 5)].

Table 4. Cross-tabulation and P-values from univariable statistics of *Staphylococcus aureus* and *Streptococcus agalactiae* PCR positivity in milk samples from 287 right rear quarters[1]

Variable	Level	N	*Staph. aureus* milk[2]			*Strep. agalactiae* milk[2]		
			# positive	% positive	P-value univariable statistics	# positive	% positive	P-value univariable statistics
Overall		287	29	10.1		40	14.0	
Staph. aureus on teat skin[2]	Negative	245	27	11.0	0.45	36	14.7	0.42
	Positive	42	2	4.8		4	9.5	
Strep. agalactiae on teat skin[2]	Negative	236	28	11.9	0.24	24	10.2	0.003
	Positive	51	1	2.0		16	31.7	
Herd	H1	28	6	21.4	NA[3]	1	3.6	NA
	H2	36	2	5.6		0	0.0	
	H3	38	1	2.6		5	13.2	
	H4	35	0	0.0		18	51.4	
	H5	37	2	5.4		3	8.1	
	H6	40	1	2.5		3	7.5	
	H7	34	3	8.8		7	20.6	
	H8	39	14	35.9		3	7.7	
Hyperkeratosis score	1 (no ring)	20	0	0.0	0.067[4]	2	10.0	0.17
	2 (smooth ring)	201	17	8.5		22	11.0	
	3 (rough ring)	48	9	18.8		11	22.9	
	4 (very rough)	18	3	16.7		5	27.8	
Parity	1	55	9	16.4	0.12	2	3.6	0.031
	2	95	10	10.5		16	16.8	
	≥ 3	137	10	7.3		22	16.1	
DIM	< 90	73	3	4.1	0.18	7	9.6	0.31
	90 – 199	83	10	12.1		13	15.7	
	≥ 200	131	16	12.2		20	15.3	
SCC (× 1,000 cells/mL)[5]	200 – 399	119	11	9.2	0.73	9	7.6	0.048
	400 – 999	95	9	9.5		20	21.1	
	≥ 1,000	73	9	12.3		11	15.1	
Time since last milking[6]	± 0.5 h	22	1	4.6	0.8	1	4.6	0.46
	0.5 h to 2.5 h	64	4	6.3		6	9.4	
	2.5 h to 5 h	66	8	12.1		11	16.7	
	5 h to 9 h	69	6	8.7		10	14.5	
	≥ 9 h	66	10	15.2		12	18.2	
CNS on teat skin[2]	Negative	139	7	5.0	0.018	12	8.6	0.015
	Positive	148	22	14.9		28	18.9	
CNS in milk[2]	Negative	107	11	10.3	0.99	10	9.4	0.33
	Positive	180	18	10.0		30	16.7	
Staph. aureus in milk[2]	Negative	258	NA	NA	NA	37	14.3	0.71
	Positive	29	NA	NA		3	10.3	
Strep. agalactiae in milk[2]	Negative	247	26	10.5	0.84	NA	NA	NA
	Positive	40	3	7.5		NA	NA	
Other major pathogens in milk[2,7]	Negative	219	21	9.6	0.57	30	13.7	0.53
	Positive	68	8	11.8		10	14.7	

[1] One quarter per cow was selected for PCR; thereby the distribution of parity, DIM and SCC represented the sampled population at the animal level. [2] PCR positive: cycle threshold ≤ 37. [3] NA = not applicable. [4] Hyperkeratosis scores 1 and 2 were merged for univariable statistics due to zero positive *Staph. aureus* quarters (outcome variable) in score 1. [5] SCC from last milk recording 5 to 33 d before sampling. [6] Time since last milking, calculated based on estimated sampling time and milking time from AMS. [7] Including *Escherichia coli*, *Enterococcus* spp., and *Lactococcus lactis* subsp. *lactis*, *Klebsiella* spp. (*pneumoniae, oxytoca* and *variicola*), *Mycoplasma* spp., *Streptococcus dysgalactiae* and *Streptococcus uberis*.

Table 5. Results from the multivariable logistic regression model of association between *Streptococcus agalactiae* PCR positivity in quarter milk and teat skin samples from 287 right rear quarters

Variable	Level	Estimate	SE	OR	95% CI		P-value
Strep. agalactiae on teat skin	Negative	0		Referent			
	Positive	1.33	0.5	3.76	1.4	10.1	0.0089
Hyperkeratosis score	1 (no ring)	0		Referent			
	2 (smooth ring)	-0.13	0.96	0.88	0.13	5.8	0.89
	3 (rough ring)	0.81	1.01	2.25	0.31	16.3	0.42
	4 (very rough)	0.9	1.17	2.46	0.24	24.8	0.44
Parity	1	0		Referent			
	2	1.79	0.86	5.97	1.1	32.4	0.039
	3+	1.84	0.85	6.3	1.19	33.4	0.031
SCC (× 1,000 cells/mL)	200 – 399	0		Referent			
	400 – 999	1.13	0.52	3.09	1.11	8.62	0.031
	≥ 1,000	0.71	0.57	2.04	0.67	6.23	0.21
CNS on teat skin	Negative	0		Referent			
	Positive	1.1	0.52	2.99	1.07	8.4	0.037

Discussion

To the best of our knowledge, this is the first study to demonstrate that teat skin colonization with *Staph. aureus* and *Strep. agalactiae* can be associated with IMI in AMS, where the transmission of bacteria during milking likely differs from the conventional milking system. In AMS milkers' hands are not a source of bacteria, but the hygiene in AMS is challenged due to the standardized cleaning process of teats before and after milking (Dohmen et al., 2010).

The odds of a quarter having *Staph. aureus* IMI were higher when teat skin colonization was detected using bacterial culture as the diagnostic method; however, we were not able to demonstrate the same association between teat skin colonization and IMI with *Staph. aureus* using a PCR test. In contrast, the odds of a quarter having *Strep. agalactiae* IMI were higher when *Strep. agalactiae* was detected on teat skin by PCR, yet when bacterial culture was used, we only isolated *Strep. agalactiae* from the teat skin of 2 cows that also had *Strep. agalactiae* IMI.

As we investigated cows at "high risk" with SCC >200,000 cells/mL from *Strep. agalactiae*-positive herds, the recorded prevalence is not comparable to that of the general population.

Staphylococcus aureus

The association between positive teat skin samples detected by culture and IMI with *Staph. aureus* in herds with AMS can be interpreted in 2 ways: IMI leads to contaminated teats, or contaminated or colonized teats lead to IMI. Whereas none of the 2 is more obvious than the other, we will discuss the findings in this light. First, a significant number of samples were both PCR and culture positive in teat samples without a simultaneous IMI. Therefore, many of these are considered colonized or contaminated from another source than the milk from the same quarter.

In line with our findings, an association between teat skin colonization and IMI with *Staph. aureus* was previously reported in conventional milking systems (Haveri et al., 2008; da Costa et al., 2014). da Costa et al. (2014)) calculated a relative risk of 4.5 for quarters being diagnosed with *Staph. aureus* IMI if the teat skin was colonized with *Staph. aureus*. The study was based on a quarter-level *Staph. aureus* prevalence of 12% for milk and 11% for teat skin, which is slightly higher than the prevalence we found (8.1 and 6.6%). However, da Costa et al. (2014)) selected the cows equally across 3 groups with different *Staph. aureus* IMI status: known infected, known uninfected, and unknown infection status, whereas our cows were randomly selected among cows with high SCC. Furthermore, da Costa et al. (2014)) covered the whole teat with the teat skin swab and included an enrichment step before plating, which likely increased the sensitivity of the bacterial culture and might explain the higher proportion of positive teat skin samples. In a study by Haveri et al. (2008)), the proportion of *Staph. aureus* IMI at the quarter level was 3 to 7% in the 2 herds included; however, *Staph. aureus* was isolated from 25 to 68% of the teat wall and teat orifice samples after including an enrichment step of the nonmilk samples. Pulsed-field gel electrophoresis analysis was also included by Haveri et al. (2008)) and da Costa et al. (2014)), and the same pulsotypes were found in milk and nonmilk samples.

In contrast, Zadoks et al. (2002)) concluded that the teat skin was not an important reservoir of IMI, as they found that different pulsotypes were significantly associated with the site of isolation (milk or teat skin). The contradictory results could be explained by the different approaches; Zadoks et al. (2002)) associated pulsotypes of 225 isolates across 43 different herds to the isolation sites, and Haveri et al. (2008)) and da Costa et al. (2014)) compared pulsotypes across isolation sites at the quarter, cow, and herd level. As discussed by Klaas and Zadoks (2018)), the heterogeneity of bacterial strains within and across herds could explain why different studies show different results. Due to limited resources, we did not investigate genotypes in the current study. Therefore, we cannot make sure that the same genotypes were present in milk and teat skin of the same quarter, and this aspect should ideally be investigated further. Nonetheless, it would not be possible to type isolates that are not present, so the origin of bacteria on teats from non-IMI cows would generally have to origin from other cows. The concentrations may not be sufficient to cause an IMI, but this cannot be determined, as we do not know the infectious dose.

In contrast to the bacterial culture results, we were not able to show an association between teat skin and milk samples tested by PCR. The PCR data set was a subset of the culture data set, yet the proportion of both milk and teat skin samples positive for *Staph. aureus* with the PCR test was higher (10 and 16%) than with bacterial culture (8.1 and 6.6%). Furthermore, a higher proportion of quarters not detected with IMI had a PCR positive teat skin sample (calculated from Table 2, Table 4). This could either be due to lower analytical and diagnostic test sensitivity of bacterial culture or lower diagnostic specificity of the PCR test, because the PCR assay may detect viable as well as nonviable and growth-inhibited cells, whereas culture is dependent exclusively on isolation of viable bacterial cells (Koskinen et al., 2009; Mahmmod et al., 2013b). The main challenge in the applied culture protocol was that most teat skin samples (Table 2) contained CNS and the reading was in some cases problematic. This may have led to a low sensitivity of bacterial culture on teat skin samples, which could have been improved by an enrichment step or dilution series of teat skin samples. On the other hand, the PCR test has not been validated for teat skin

samples, and the specificity is not known. Furthermore, we do not know how postmilking teat disinfection products might affect teat skin samples and inhibit bacterial growth in bacterial culture without influencing detection using the PCR method.

Like the studies of Haveri et al. (2008)) and da Costa et al. (2014)), our study is cross-sectional in nature, thus no directionality can be assessed. It is therefore possible that the association is related to contamination of the teat skin by milk containing *Staph. aureus*. That could also be an explanation of the contradictory results from culture and PCR, if the concentration and viability of *Staph. aureus* possible to detect by bacterial culture is only sufficient if it originates from milk of the same cow or quarter, whereas what is detected by PCR is also nonviable or low concentrations of *Staph. aureus* obtained from the environment, milking equipment, and other cows.

Streptococcus agalactiae

We isolated viable *Strep. agalactiae* from the teat skin of 2 dairy cows with subclinical mastitis, milked in AMS. To our knowledge, this is the first time *Strep. agalactiae* have been isolated from teat skin since the reporting by Chodkowski (1949)). This result is in line with findings from several other studies conducted in recent years, which state that *Strep. agalactiae* is able to survive in the environment and can be isolated from extramammary body sites (Jørgensen et al., 2016; Farre et al., 2017; Henriksen et al., 2017). However, calculating an association between teat skin colonization and IMI was not possible due to the low number of positive teat skin samples (n = 4). We therefore cannot exclude that there may be an association when investigating a population with a higher prevalence of *Strep. agalactiae*. The prevalence reported by Chodkowski (1949)) was 38%, and the much lower prevalence (0.35%) found in our study may indicate that *Strep. agalactiae* is not very likely to colonize teat skin. The culture methods differed as Chodkowski (1949)) used sterile milk for an enrichment step before plating, likely increasing the sensitivity of bacterial culture compared with our method. Furthermore, the population prevalence could have been much higher in 1949 compared with now. It is, however, more likely that the few quarters we detected as positive on teat skin were contaminated by milk of the same quarter also *Strep. agalactiae* culture positive.

In contrast to the culture results, an association between *Strep. agalactiae* on teat skin and in milk was observed using PCR, suggesting that teat skin should be considered as a reservoir for *Strep. agalactiae*. In addition, *Strep. agalactiae* was detected using PCR on the teat skin of quarters without IMI (Table 4), which is comparable to observations of *Staph. aureus* (Haveri et al., 2008; Piccinini et al., 2009; da Costa et al., 2014). A positive result for *Strep. agalactiae* or *Staph. aureus* using the PCR assay on nonaseptically collected milk samples could therefore represent contamination from the teat skin instead of a true IMI, thus potentially leading to an incorrect diagnosis (Piccinini et al., 2009; da Costa et al., 2014).

Furthermore, the proportion of *Strep. agalactiae*-positive teat skin samples detected by PCR (18%) was considerably larger than when bacterial culture was used (0.35%), indicating a higher test sensitivity or lower test specificity of the PCR test compared with bacterial culture, with the same arguments as mentioned for *Staph. aureus*. Likewise, the prevalence of *Strep. agalactiae* IMI was higher with PCR (14%) compared with bacterial culture (7.4%). Polymerase chain reaction

has previously been reported as more sensitive than bacterial culture in relation to the detection of *Strep. agalactiae* IMI (Mahmmod et al., 2013b). As for *Staph. aureus*, the PCR test detecting lower concentrations of bacteria and probably nonviable bacteria on teat skin may likely be the explanation. With regard to the cross-sectional study design, we cannot make sure that teat skin colonization caused the IMI, but it appears that *Strep. agalactiae* and *Staph. aureus* in some stage of viability and some level of concentration are circulating in the milking system or surrounding barn environment, making it possible to detect the bacteria on the teat skin, especially with PCR. This may be a method of transmission and hygiene, including dirty teats before milking and bad coverage of postmilking teat disinfectant, has previously been associated with udder health in AMS (Dohmen et al., 2010). Therefore, we suggest that reduction of transmission may occur if (1) teats are cleaned before milking, (2) postmilking teat disinfectant is applied with good coverage after milking, and (3) clusters are cleaned between each cow.

Conclusions

Our bacterial culture findings showed that teat skin colonization with *Staph. aureus* resulted in a 7.8 (2.9; 20.6) times higher odds of *Staph. aureus* IMI in cows with SCC >200,000 cells/mL in herds with AMS and a history of bulk milk positive for *Strep. agalactiae*. In contrast, results from a PCR assay on a subset of samples did not support this association, but *Staph. aureus* was detected in teat skin and milk with both PCR and bacterial culture. *Streptococcus agalactiae* was also detected in teat skin samples with both PCR and bacterial culture, yet the number of positive teat skin samples detected by culture was too low to carry out further analysis on the association between teat skin colonization and IMI. Results from the PCR assay showed that quarters with *Strep. agalactiae* on teat skin had a 3.8 (1.4; 10.1) times higher odds of *Strep. agalactiae* IMI. We conclude that the presence of *Staph. aureus* and *Strep. agalactiae* on teat skin may be a risk factor for IMI with the same pathogens and focus on teat skin hygiene is still recommendable; however, no causal relation can be established.

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Manuscript II

Accuracy of qPCR and bacterial culture for the diagnosis of bovine intramammary infections and teat skin colonisation with *Streptococcus agalactiae* and *Staphylococcus aureus* using Bayesian analysis

Line Svennesen,[1*] Yasser S. Mahmmod,[1,2†] Nanna K. Skjølstrup,[1] Louise R. Mathiasen, [1] Jørgen Katholm,[3] Karl Pedersen, [4] Ilka C. Klaas[1‡] and Søren S. Nielsen,[1]

[1]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
[2]Infectious Diseases, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia Province, Egypt
[3]DNA Diagnostic A/S, Voldbjergvej 14, 8240 Risskov, Denmark
[4]National Veterinary Institute, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
[†]Current address: IRTA, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona, 08193-Bellaterra, Barcelona, Spain.
[‡]Current address: DeLaval International AB, Tumba, Sweden 14741.

*Corresponding author: Line Svennesen, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegårdsvej 2, DK-1870 Frederiksberg C, Denmark. Email: line.svennesen@sund.ku.dk. Phone: +45-35331987

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Abstract

Streptococcus agalactiae (*Strep. agalactiae*) and *Staphylococcus aureus* (*Staph. aureus*) are originally regarded as contagious mastitis pathogens, however, both pathogens have recently been isolated from extramammary and environmental sites, indicating that other sites than the udder might contribute to the spread of these pathogens potentially causing intramammary infections. Diagnostic tools to identify pathogens at extramammary sites are available but still needs to be validated. The objective of this cross-sectional field study was to estimate the diagnostic sensitivity (Se) and specificity (Sp) of the commercially available Mastit4 qPCR assay and bacterial culture (BC) in identifying *Strep. agalactiae* and *Staph. aureus* from milk and teat skin samples. We randomly selected 30–40 cows with high somatic cell counts from eight Danish *Strep. agalactiae*-positive dairy herds with automatic milking systems. Teat skin samples and aseptic milk samples were collected from right rear quarters (n = 287) for BC and PCR analysis. Se and Sp were estimated in a Bayesian latent class analysis. For milk samples, the Se and Sp of qPCR for *Strep. agalactiae* were estimated to 0.97 and 0.99, respectively, whereas the Se and Sp of BC were 0.41 and 1.00, respectively. The Se and Sp of qPCR for *Staph. aureus* were estimated to 0.95 and 0.99, respectively, whereas the Se and Sp of BC were 0.54 and 0.77, respectively. For teat skin samples, the Se and Sp of qPCR for *Strep. agalactiae* were estimated to be 0.97 and 0.96, respectively, whereas the Se and Sp of BC were 0.33 and 1.00, respectively. The Se and Sp of qPCR for *Staph. aureus* were estimated to 0.94 and 0.98, respectively, whereas the Se and Sp of BC were 0.44 and 0.74, respectively. In conclusion, the Se for diagnosing *Strep. agalactiae* and *Staph. aureus* IMI was higher for qPCR than BC, suggesting that qPCR is a valuable method for detecting both pathogens from quarter-level milk samples. The performance of BC in the detection of *Strep. agalactiae* and *Staph. aureus* on teat skin was poor compared to qPCR, indicating that differences in the target condition of the two methods should be considered when implementing them as routine diagnostic tests for detecting teat skin colonisers. The low Se of BC may preclude the use of BC for skin testing, and qPCR is better for this task.

Key words: Latent class analysis, Mastitis, Polymerase chain reaction, Sensitivity, Specificity

Introduction

Intramammary infections (IMI) are a major economic and public health challenge in dairy herds (Keefe, 2012), and the use of antibiotics for mastitis treatment constitutes the majority of the total usage in Danish dairy herds (DANMAP, 2016). Therefore, effective control of mastitis is an important factor in reducing the risk of antimicrobial resistance.

The contagious udder pathogen *Staphylococcus aureus* (*Staph. aureus*) is widespread in dairy herds, and despite successful control efforts to reduce *Streptococcus agalactiae* (*Strep. agalactiae*) in Scandinavian countries during the 20th century (Katholm et al., 2012; Lyhs et al., 2016), the proportion of positive herds in Denmark and Norway increased throughout the early 21st century (Katholm et al., 2012; Mweu et al., 2012; Radtke et al., 2012). The estimated herd-level prevalence of *Staph. aureus* and *Strep. agalactiae* in Denmark, Germany, Belgium and Canada has been reported at 91% and 7% (Katholm et al., 2012), 90% and 29% (Tenhagen et al., 2006), 86% and 5.3% (Piepers et al., 2007), and 74% and 1.6% (Olde Riekerink et al., 2006), respectively.

Although both bacteria are considered contagious pathogens, environmental reservoirs have been described in the scientific literature (Haveri et al., 2008; Jørgensen et al., 2016). Klaas and Zadoks (2017) added that a faeco-oral transmission cycle may perpetuate and amplify the presence of *Strep. agalactiae* within dairy herds, but the importance of these environmental reservoirs is still being discussed. Furthermore, it has yet to be determined whether it is primarily milk that is contaminating the environment, or if colonisation of extramammary sites also leads to IMI.

Advances in the dairy industry have led to larger herds, and automatic milking systems (AMS) became more frequent in the Nordic countries in the early 21st century (Barkema et al., 2015). Milking hygiene and teat cleaning in AMS differ from the conventional milking system, with more cows per milking unit and no contact with human hands (Hovinen and Pyörälä, 2011; Rodenburg, 2017). This, together with the environmental reservoir of contagious mastitis pathogens, could explain why *Staph. aureus* remains a problem, and why there has been a re-emergence of *Strep. agalactiae* in line with an increase in the proportion of farms using AMS in Denmark (Bennedsgaard and Katholm, 2013).

Accurate diagnostic tests to detect pathogen-specific subclinical mastitis are essential in initiating appropriate control efforts (Barkema et al., 2006; van den Borne et al., 2010), e.g., separating infected from susceptible animals and establishing other measures to reduce the risk of spread within and between herds (Barkema et al., 2009). Bacterial culture (BC) has been considered the reference standard for identifying mastitis pathogens, but studies comparing the sensitivity (Se) and specificity (Sp) of real-time PCR and BC for diagnosing IMI with *Staph. aureus* or *Strep. agalactiae* in composite milk samples have suggested a higher Se for PCR compared to BC (Holmöy et al., 2018; Mahmmod et al., 2013a, 2013b). Furthermore, the bovine teat skin may be an important reservoir for contagious mastitis pathogens, as the presence of bacteria on teat skin has been associated with IMI in the same quarter and bacteria has been found on teat skin in quarters not having IMI, suggesting that colonisation or contamination of teat skin from sources other than milk of the same quarter is likely (da Costa et al., 2014; Svennesen et al., 2018). Furthermore, controlling *Staph. aureus* and *Strep. agalactiae* in large herds without considering

the environmental reservoirs may lead to unsuccessful control and eradication. Therefore, PCR tests on teat skin or environmental samples could become a useful tool in controlling *Strep. agalactiae* and *Staph. aureus* mastitis.

The objective of this cross-sectional field study was to estimate the Se and Sp of the commercially available Mastit4 qPCR assay and BC for the identification of *Strep. agalactiae* and *Staph. aureus* in milk and teat skin samples from high somatic cell count (SCC) cows in AMS herds positive for *Strep. agalactiae*. In the absence of a reference test, a Bayesian latent class analysis (LCA) framework was used.

Materials and methods

We followed the guidelines for reporting diagnostic accuracy in studies that use Bayesian LCA (Kostoulas et al., 2017).

Study population

Eight dairy herds with Danish Holstein cows were selected for a project investigating the epidemiology and diagnostics of *Strep. agalactiae*, *Staph. aureus* and non-aureus staphylococci in Danish AMS herds. Eligible herds had ≥ 3 milking robots and a bulk tank milk PCR cycle threshold (Ct) value ≤ 32 for *Strep. agalactiae*. More herd characteristics are presented in Mahmmod et al. (2018). Samples were collected from the right rear quarters of 30 to 40 lactating dairy cows from each herd. These cows were randomly selected among those with a SCC > 200,000 cells/mL at the preceding milk recording, and with no clinical mastitis or antimicrobial treatment four weeks prior to sample collection.

Sample collection

The cows selected for sampling were separated by the farmer and restrained in headlocks during sampling. Before sampling, the cows followed their normal milking routine, meaning that the time since last milking varied from 30 min to approximately 12 h. All herds used post-milking teat disinfection in the AMS (Mahmmod et al., 2018).

The teats were cleaned with dry paper towels using at least one for each teat until they were visually clean. The teat skin samples were taken with the modified wet-dry method (Paduch and Kroemker, 2011) using a wet and a dry rayon swab (DaklaPack, Glostrup, Denmark) for each teat. The swabs were rolled 360° around the teat about 1 cm from the teat canal orifice and were then broken into a corresponding tube containing 2 mL of ¼ Ringer's solution (Merck, Darmstadt, Germany).

Quarter-level milk samples were collected directly after the teat skin swab samples, according to the National Mastitis Council guidelines (NMC, 1999). In brief, the teat end was disinfected with cotton swabs soaked in ethanol (70%). Individual quarter foremilk samples were then aseptically collected in sterile screw-cap plastic tubes. Latex gloves were worn and were changed after each cow and sampling procedure. Tubes containing the teat skin and milk samples were stored at a maximum of 5 °C and delivered to the microbiology laboratory within 24 h. All study activities including farm visits, collection of samples and laboratory examination were carried out between February and May 2017.

Bacterial culture

Milk samples were vortexed for 10 s, and 10 µL was streaked with a disposable calibrated loop onto a quarter of a plate of each a calf blood agar (5% sheep blood), a chromogenic agar selective for staphylococci (SASelect, Bio-Rad, Hercules, CA) and a modified Edward's medium (Oxoid, Roskilde, Denmark) supplemented with 5% calf blood and 2% filtrate of a β-toxin producing *Staph. aureus*, prepared as described by Jørgensen et al. (2016).

Teat skin samples were vortexed for 20 s, and the swabs were removed with a sterile pair of tweezers before 100 µL of the sample was inoculated and spread with a Drigalski spatula on a whole plate of calf blood agar, SASelect medium and modified Edward's medium. The plates were incubated aerobically at 37 °C for 48 h in total and read after 24 and 48 h. The approximate number of colony forming units (cfu) was determined by colony counting.

Colonies of *Strep. agalactiae* were phenotypically identified on blood agar and modified Edward's medium. Suspected colonies were confirmed as *Strep. agalactiae* using latex agglutination for Lancefield group B (PathoDxtra Strep Grouping Kit, ThermoFisher Scientific, Waltham, MA) or MALDI-TOF (Bruker Biotyper software system, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany). *Staph. aureus* colonies were phenotypically identified on SASelect medium according to the manufacturer's guidelines, and on calf blood agar according to the National Mastitis Council recommendations (NMC, 1999). Suspected colony types were confirmed as *Staph. aureus* using MALDI-TOF. For BC, a quarter was defined as positive in milk or teat skin if at least one colony of *Staph. aureus* or *Strep. agalactiae* appeared on any of the used agar plates. This corresponded to a detection limit of 100 cfu/mL for milk and 10 cfu/mL for teat skin samples.

qPCR assay

A FLOQswab (COPAN ITALIA spa, Brescia, Italy) was immersed in the original milk and teat skin samples immediately after streaking for BC. The principle of using these swabs were that they would dry out quickly, thus there was no need for cooling under transport. The swabs were shipped to the laboratory of DNA Diagnostic A/S on the same day that BC was performed, for analysis 1 or 2 days later.

The samples were tested using the Mastit4 qPCR assay (DNA Diagnostic, Risskov, Denmark). The personnel at the laboratory were blinded to the samples and results of the BC. Ct values were recorded for each sample, and samples were defined as positive for *Staph. aureus* or *Strep. agalactiae* if the Ct value was ≤ 37.

The approximate volume soaked by the FLOQSwab was 220 µl, and after DNA extraction and purification steps, this left 18 µl of the original sample for qPCR analysis, corresponding to 60% of the 3 × 10 µl spread on plates for milk samples and 6% of the 3 × 100 µl inoculated on plates for teat skin samples.

Statistical analysis

In the absence of a reference standard to classify true cases of IMI and teat skin colonisation with *Strep. agalactiae* and *Staph. aureus*, the test characteristics (Se and Sp) of qPCR and BC were

estimated using a Bayesian LCA model (Branscum et al., 2005) based on the paradigm described by Hui and Walter (1980).

The study population was divided into two subpopulations based on robot type used in the herds from which the cows originated. Robot type could be considered a risk factor, thus different herd prevalences were expected. Priors for *Staph. aureus* were used based on the results from Mahmmod et al. (2013a), and we considered these to be informative despite them being based on composite milk samples and the PCR test being from another manufacturer. Priors for *Strep. agalactiae* (Se and Sp of BC and PCR) were based on the results from Holmøy et al. (2018), who used the same PCR test, but composite milk samples. No prior information on the diagnostic performance of BC or qPCR on teat skin samples was available, so non-informative priors and the priors from milk were used. All priors are given in Table 1. For non-informative priors, a Beta(1,1) distribution was used. For informative priors, the particular prior distribution was created based on the median and the 95% posterior credibility interval (PCI) reported in the original publications with the Beta-distributions shown in Table 1.

Table 1. Priors used for analysis: Median and 95% posterior credible interval (PCI) for sensitivity (SeqPCR) and specificity (SpqPCR) estimates for qPCR and sensitivity (SeBC) and specifity (SpBC) estimates for bacterial culture for *Streptococcus agalactiae* (Holmøy et al., 2018) and *Staphylococcus aureus* (Mahmmod et al., 2013a) in composite milk samples.

Pathogen	Parameter	Test estimates		Probability distribution
		Median	95% PCI	
Strep. agalactiae	SeqPCR	0.93	0.78 - 1.00	Beta(16.3; 1.2)
	SeBC	0.39	0.32 - 0.47	Beta(78; 121)
	SpqPCR	0.99	0.95 - 1.00	Beta(94; 1.46)
	SpBC	1.00	0.99 - 1.00	Beta(365; 1.36)
Staph. aureus	SeqPCR	0.91	0.74 - 1.00	Beta(14.3; 1.55)
	SeBC	0.52	0.44 - 0.61	Beta(72; 67)
	SpqPCR	0.99	0.94 - 1.00	Beta(71; 1.35)
	SpBC	0.90	0.86 - 0.94	Beta(138; 114)

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. Three chains were run and 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 the initial burn-in period was assessed by visual inspection of the time-series plots of the chains. Posterior inference was based on median and 95% PCI for the Se and Sp of the two tests, where the PCI was constructed based on the percentiles of the posterior distributions. An example of the Open Bugs code is available in Appendix I.

Results

In total, 287 quarters with complete observations for qPCR and BC from milk and teat skin samples were used for the LCA analysis. Results of cross-tabulation (contingency table) of the dichotomous outcome of qPCR and BC for the detection of *Strep. agalactiae* and *Staph. aureus* from Population 1 (robot type 1) and Population 2 (robot type 2) are displayed in Table 2.

Estimates of the posterior median and 95% PCI of Se and Sp of qPCR and BC for the detection of *Strep. agalactiae* and *Staph. aureus* are displayed in Table 3.

Table 2. Cross-tabulated results for combinations of qPCR at a Ct value cut-off ≤ 37 and bacterial culture (BC) at a cut-off ≥ 1 cfu for identification of *Streptococcus agalactiae* and *Staphylococcus aureus* from 287 quarter-level milk and teat skin samples collected from eight Danish dairy herds with AMS, stratified based on robot type (Population 1 = 6 herds, Population 2 = 2 herds).

Sample	Population	Pathogen	Test combinations (T1; qPCR and T2; BC)				Total
			T1+/T2+	T1+/T2-	T1-/T2+	T1-/T2-	
Milk	Population 1 (Robot type 1)	*Strep. agalactiae*	10	20	0	184	214
		Staph. aureus	7	5	1	201	214
	Population 2 (Robot type 2)	*Strep. agalactiae*	9	1	0	63	73
		Staph. aureus	12	5	2	54	73
Teat skin	Population 1 (Robot type 1)	*Strep. agalactiae*	1	47	0	166	214
		Staph. aureus	5	35	8	166	214
	Population 2 (Robot type 2)	*Strep. agalactiae*	0	3	0	70	73
		Staph. aureus	2	0	8	63	73

For milk, the posterior median Se estimates for *Strep. agalactiae* were 0.97 and 0.41 for qPCR and BC, respectively, with PCI as shown in Table 3. The corresponding Sp estimates were 0.99 and 1.00, using the results from the analysis with informative priors. For *Staph. aureus*, the median Se estimates were 0.95 and 0.54 for qPCR and BC, respectively, with corresponding Sp estimates of 0.99 and 0.77. For teat skin, Se for *Strep. agalactiae* were 0.97 for qPCR and 0.33 for BC, and the corresponding Sp estimates were high (0.96 and 1.00). For *Staph. aureus*, Se estimates were 0.94 and 0.44 for qPCR and BC, respectively, with corresponding Sp estimates of 0.98 and 0.74. The sensitivity analyses demonstrated that most estimates were relatively unaffected by the choice of priors, except for the sensitivity of BC, which appeared to be affected to some extent (Table 3).

Table 3. Posterior median and 95% posterior credible interval (PCI) of sensitivity (SeqPCR) and specificity (SpqPCR) for Mastit4 qPCR assay at a Ct value cut-off ≤ 37, and sensitivity (SeBC) and specificity (SpBC) for bacterial culture at a cut-off of ≥ 1 cfu of *Streptococcus agalactiae* and *Staphylococcus aureus* in quarter-level milk and teat skin samples from 287 cows in eight Danish dairy herds with AMS.

Sample	Pathogen	Parameter	Test estimates Informative priors[1]		Test estimates Non-informative priors	
			Median	95% PCI	Median	95% PCI
Milk	*Strep. agalactiae*	SeqPCR	0.97	0.88 – 1.00	0.96	0.82 – 1.00
		SeBC	0.41	0.35 – 0.47	0.82	0.44 – 0.99
		SpqPCR	0.99	0.97 – 1.00	0.93	0.89 – 0.99
		SpBC	1.00	0.99 – 1.00	1.00	0.99 – 1.00
	Staph. aureus	SeqPCR	0.95	0.82 – 1.00	0.88	0.68 – 0.99
		SeBC	0.54	0.46 – 0.62	0.74	0.52 – 0.96
		SpqPCR	0.99	0.96 – 1.00	0.98	0.95 – 1.00
		SpBC	0.77	0.73 – 0.81	0.99	0.98 – 1.00
Teat skin	*Strep. agalactiae*	SeqPCR	0.97	0.87 – 1.00	0.23	0.0041 – 0.95
		SeBC	0.33	0.27 - 0.41	0.0092	0.00030 – 0.071
		SpqPCR	0.96	0.89 – 1.00	0.91	0.06 – 1.00
		SpBC	1.00	0.99 – 1.00	0.99	0.93 – 1.00
	Staph. aureus	SeqPCR	0.94	0.80 – 0.99	0.077	0.0022 – 0.90
		SeBC	0.44	0.36 – 0.52	0.087	0.030 – 0.26
		SpqPCR	0.98	0.94 – 1.00	0.82	0.08 – 1.00
		SpBC	0.74	0.70 – 0.78	0.91	0.72 – 0.97

[1] The used priors are shown in Table 1

Discussion

This study estimated the test accuracy of BC and the commercially available Mastit4 qPCR assay using LCA, which does not require a perfect reference test. To our knowledge, this is the first study to evaluate BC and qPCR assays on quarter-level milk and teat skin samples for detection of *Staph. aureus* and *Strep. agalactiae*.

In general, we found a higher Se of qPCR compared to BC across pathogen and sample type. The Sp of BC and qPCR were at the same level for *Strep. agalactiae*, but for *Staph. aureus* the Sp of BC was lower than for qPCR.

The considerable differences in the performance of BC and qPCR is probably due to that the two tests have different target conditions; viable bacteria vs. bacterial DNA, which subsequently could affect the test performance. This particular aspect should be considered for clinical application, interpretation, and future investigation.

Estimates of qPCR and BC for IMI

The higher Se of qPCR compared to BC is in line with the general perception that qPCR is more sensitive than BC (Holmøy et al., 2018; Mahmmod et al., 2013a, 2013b; Nyman et al., 2016).

In the current study, the Sp of BC and qPCR were comparable for *Strep. agalactiae*, but for *Staph. aureus*, the Sp of BC was lower than for qPCR. Holmøy et al. (2018) also reported similar Sp

estimates for BC and qPCR for *Strep. agalactiae*, and Mahmmod et al., 2013a, Mahmmod et al., 2013b reported lower Sp of BC compared to qPCR for *Staph. aureus*, but also for *Strep. agalactiae*, whereas Nyman et al. (2016) and Cederlöf et al. (2012) reported comparable estimates for the Sp of BC and qPCR for *Staph. aureus*.

These differences could be explained by the different sampling and laboratory procedures. In the current study, we evaluated both qPCR and BC on the same aseptic quarter-level milk sample, whereas previous studies (Cederlöf et al., 2012; Mahmmod et al., 2013a, 2013b; Nyman et al., 2016) evaluated a different PCR assay (PathoProof Mastitis PCR assay) on non-aseptically collected composite milk samples and compared this to results from BC on aseptic quarter-level milk samples.

Koskinen et al. (2009) estimated the analytical Se and Sp of the PathoProof Mastitis PCR assay at 100% for identifying bacteria from isolates originating from bovine mastitis. Rattenborg et al. (2015) found moderate-to-high agreement between the PathoProof Mastitis PCR assay and Mastit4 qPCR assay for *Strep. agalactiae* in bulk tank milk samples, whereas the agreement for *Staph. aureus* was moderate (Ct value cut-off ≤ 37).

Using different samples for the two tests increases the risk of not having the same concentration of bacteria (bacterial load), and variation in shedding has been demonstrated for both *Staph. aureus* and *Strep. agalactiae* (Thieme and Haasmann, 1978; Sears et al., 1990). Furthermore, non-aseptically collected milk samples may increase the number of false positive samples due to contamination and carryover (Mahmmod et al., 2017), and evaluating BC on quarter-level against PCR on composite milk samples could also decrease Se of PCR due to dilution of the sample (unless all four quarters are infected). Furthermore, the selection of cows may influence the results, especially the prevalence in the investigated population. We selected cows with SCC > 200,000 cells/mL which may not only increase the frequency of IMI, but also the test performance due to an increased chance of a high concentration of bacteria in IMI quarters with an active infection. This could have led to the higher Sp of PCR, where non-viable bacteria have a relatively minor influence compared to a setup with random selected cows.

Our estimates for *Strep. agalactiae* in milk samples fell within the range reported by Mahmmod et al. (2013b) and Holmöy et al. (2018). For *Staph. aureus*, our estimates fell within the range reported by Mahmmod et al. (2013a), while our estimates of Se and Sp for BC were lower (0.54 and 0.77, respectively) than those reported by Cederlöf et al. (2012; 0.83 and 0.97, respectively). This may be explained by the use of results from Mahmmod et al. (2013a) as informative priors, which seem to highly influence the estimates because of less robust estimates due to the relatively small sample size and low number of positive BC test results (Table 2).

Estimates of qPCR and BC for teat skin colonisation

As there were no priors for teat skin samples available, the current teat skin results may serve as priors for future studies. Like for milk samples, the estimates were highly influenced by the informative priors used, primarily increasing the Se estimates of both BC and qPCR (Table 3).

The Sp estimates were generally high, except the Sp of BC for *Staph. aureus* (informative priors). Using non-informative priors resulted in very low Se of both BC and qPCR. When informative priors were used, the Se increased and the Se of qPCR was significantly higher than the Se of BC, even though the amount of original sample material for qPCR analysis made up only 6% of that for BC. Previous studies used BC to detect teat skin colonisation with *Staph. aureus* (Haveri et al.,

2008; da Costa et al., 2014), but overgrowth with other bacteria could challenge the Se of BC. In contrast, by using BC we ensure that the pathogens detected are potentially capable of colonising the teat skin, whereas qPCR could detect non-viable bacterial cells (Koskinen et al., 2009; Mahmmod et al., 2013b; Holmöy et al., 2018), e.g. contamination or flora of the teat skin inactivated by post-milking teat disinfection. Detection of non-viable bacteria with qPCR and a low Se of BC could explain the lower number of positive teat skin samples detected by BC for both *Staph. aureus* and *Strep. agalactiae* compared to those detected by qPCR (Table 2). However, according to the manufacturer, in the Mastit4 qPCR assay the extraction step includes that the bacteria are centrifuged to a pellet two times before the lysis procedure. This ensures that the test will not detect free DNA, meaning that the test result is only influenced to small extent by DNA from dead bacteria for which the bacteria membrane is still intact. Furthermore, PCR methods that enable distinction between live and dead bacteria have been developed (Nocker et al., 2007) and such method could be applied to environmental and teat skin samples. Result would help ruling out the issue of whether bacteria colonize or merely contaminate teat skin.

Teat skin colonisation has been associated with IMI (Haveri et al., 2008; da Costa et al., 2014; Svennesen et al., 2018), and environmental reservoirs could easily colonise the teat skin (Haveri et al., 2008; Jørgensen et al., 2016). However, the load of bacteria necessary to cause an infection is not known, and the clinical relevance of a low concentration of possibly non-viable bacteria detected by qPCR on teat skin is hard to estimate. Furthermore, as we collected samples from cows at different times since last milking, some of our samples may be more affected by post-milking teat disinfection (cows sampled just after milking), and others more by the environmental reservoir of bacteria (cows laying in cubicles before sampling). It is therefore generally difficult to assess whether it is teat skin colonisation or contamination detected in these types of samples, and the choice of test (high or low Se and Sp) should depend on the goal of the sampling.

A lower Ct value cut-off could increase Sp of the qPCR test by increasing the detection limit and not consider very low concentration of bacteria, e.g. contamination, as positive test results. However, as the Sp of qPCR for teat skin is relatively high [0.96 for *Strep. agalactiae* and 0.98 for *Staph. aureus* (informative priors)], it is unlikely that setting a lower threshold would make a substantial difference.

Model assumptions

Firstly, variation in prevalence between populations is fundamental to LCA models (Kostoulas et al., 2017; Toft et al., 2005). In this study, we used robot type to create populations with different prevalences, and the assumption was verified, as posterior estimates of prevalence were different (data not shown). Secondly, the test characteristics (Se and Sp) should be constant across the tested populations, which was the case as robot type would not affect test characteristics. Thirdly, there should be independence of tests given the target condition (i.e. the presence of pathogen or parts of the pathogen); this assumption was fulfilled because qPCR and BC have different biological identification mechanisms and no culturing was involved in the qPCR procedure.

Conclusions

The Se for diagnosing IMI with *Strep. agalactiae* and *Staph. aureus* was higher using qPCR compared to BC. This suggests that qPCR is a valuable method for detecting both pathogens from quarter-level milk samples. For testing teat skin samples qPCR also has potential due to the higher Se in the detection of *Strep. agalactiae* and *Staph. aureus* and higher Sp for *Staph. aureus*. However, the clinical importance of the findings in teat skin samples with the two different tests must be carefully evaluated, and further studies are required to reduce the uncertainty.

Declaration of interests

Our co-author Jørgen Katholm is affiliated with DNA Diagnostic A/S, which provided us with the PCR swabs and ran the qPCR analyses in their laboratory. We confirm that the laboratory personnel were blinded to the sample identification and results of bacterial culture, and that the company had no impact on the data handling or statistical analysis. Therefore, DNA Diagnostic A/S could not bias the contents of this paper.

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Appendix A. Supplementary data

The following are Supplementary data to this article:

#Open Bugs Model code with data example for *Streptococcus agalactiae* in milk using informative priors

```
model{
# Priors for Se and Sp and the prevalence (p)
for (i in 1:2){
p[i] ~ dbeta(1,1);
}
#Informative priors Strep. ag. milk and teat
se[1] ~dbeta(16.3,1.2)
se[2]~dbeta(78,121)
sp[1]~dbeta(94,1.46)
sp[2]~dbeta(365,1.36)

# The model
for (i in 1:2 ) {
pop[i,1:4] ~ dmulti(par[i,1:4],n[i]);
par[i,1] <- se[1]*se[2]*p[i] + (1-sp[1])*(1-sp[2])*(1-p[i]);
par[i,2] <- se[1]*(1-se[2])*p[i] + (1-sp[1])*(sp[2])*(1-p[i]);
par[i,3] <- (1-se[1])*(se[2])*p[i] + (sp[1])*(1-sp[2])*(1-p[i]);
par[i,4] <- (1-se[1])*(1-se[2])*p[i] + (sp[1])*(sp[2])*(1-p[i]);
n[i] <- sum(pop[i,1:4])
}
}
```

#Inits for *Streptococcus agalactiae*

```
list(se=c(0.93,0.39), sp=c(0.95,0.998), p=c(0.15,0.05))
```

Data for *Streptococcus agalactiae* milk Test 1: PCR; Test 2: BC)

```
pop[,1] pop[,2] pop[,3] pop[,4]
10 20 0 184
9 1 0 63
END
```

Manuscript III

Expert evaluation of different infection types in dairy cow quarters naturally infected with *Staphylococcus aureus* or *Streptococcus agalactiae*

Line Svennesen,[1*] Thomas B. Lund,[2] Alice P. Skarbye,[3] Ilka C. Klaas[1‡] and Søren S. Nielsen,[1]

[1]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
[2]Department of Food and Resource Economics (IFRO), Faculty of Science, University of Copenhagen, 1870 Frederiksberg C, Denmark
[3]Department of Animal Science, Aarhus University, 8830 Tjele, Denmark NA Diagnostic A/S, Voldbjergvej 14, 8240 Risskov, Denmark
‡Current address: DeLaval International AB, Tumba, Sweden 14741.

*Corresponding author: Line Svennesen, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegårdsvej 2, DK-1870 Frederiksberg C, Denmark. Email: line.svennesen@sund.ku.dk. Phone: +45-35331987

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Abstract

The purpose of this study was to improve the diagnostic recommendations for *Staphylococcus aureus* and *Streptococcus agalactiae* control using bacterial culture (BC), polymerase chain reaction (PCR) and somatic cell count (SCC) as diagnostic methods. The study was carried out in three steps: firstly, diagnostic test patterns for naturally infected quarters with *Staph. aureus* (24 quarters) and *Strep. agalactiae* (16 quarters) were created by sampling the quarters each day for 21 days and analysing the daily quarter milk samples using BC, PCR and SCC. Secondly, 30 mastitis experts were asked to group and describe the diagnostic test patterns and to establish a diagnosis for each group. The experts' statements regarding the groups they established were subsequently examined using qualitative content analysis to assign "infection types" to the statements. Lastly, the test performance was estimated for BC, PCR and SCC using generalised logistic regression models with the interpreted statements as a reference for infection.

The experts mainly identified the *Staph. aureus* quarter-patterns as persistent infections, while some had more dynamic patterns. *Strep. agalactiae* quarter-patterns mainly involved persistent infection, yet some appeared hard to diagnose and were assigned to almost all different infection types, while experts did not agree on the interpretation.

Estimates of Se for detection of *Staph. aureus* infection were 95.9% [93.7; 97.3] for BC, 99.5% [98.3; 99.8] for PCR, and 96.1% [94.0; 97.5] for SCC. The corresponding Sp estimates were 74.5% [65.7; 81.7], 66% [57.2; 73.8] and 43.7% [36.2; 51.5] for BC, PCR and SCC, respectively.

The Se estimates of BC and PCR for *Strep. agalactiae* infection were 100% [83.5; 100] and 99.9% [99.6; 100], respectively, whereas the Se of SCC detecting *Strep. agalactiae* infection was only 34.3% [26.4; 43.3]. This indicated that *Strep. agalactiae*-positive BC and PCR test results were more important than SCC results to the experts when diagnosing a quarter as infected. The Sp estimates of BC, PCR and SCC for *Strep. agalactiae* infection were 99% [72.8; 100], 97.7% [62.1; 99.9], and 65.7% [56.7; 73.7], respectively.

We conclude that PCR and BC are highly sensitive in the detection of persistent and new infections as defined by the experts, although the Se was not always 100%. An accepted lower Sp suggests that experts place less emphasis on false-positive results. We recommend that efforts are made to develop consistent terminology to characterise intramammary infections over time so that the course of infection can be taken into account at diagnosis.

Key words: contagious mastitis, sensitivity, shedding pattern, specificity

Introduction

Intramammary infections (IMI) with *Staphylococcus aureus* and *Streptococcus agalactiae* are usually associated with subclinical mastitis that reduces milk quality and production (Keefe, 2012). Even though the control of contagious mastitis in dairy herds has been a goal for many years, *Staph. aureus* is still widespread and the proportion of *Strep. agalactiae*-positive herds in Scandinavia increased early in the 21st century (Mweu et al., 2012; Radtke et al., 2012; Rainard et al., 2017).

The success of control programmes depends on our ability to detect clinical as well as subclinical cases of mastitis caused by the contagious pathogens. Diagnostic test performance, cyclic shedding patterns and daily variation in SCC can influence our accurate identification of an udder quarter as infected or non-infected.

One factor that contributes to the challenging nature of detection is the tendency for bacteria to be excreted from the infected quarters in a cyclic or fluctuating pattern, which has been shown for both *Strep. agalactiae* (Thieme and Haasmann, 1978) and *Staph. aureus* (Sears et al., 1990; Studer et al., 2008). As a consequence, repeated sampling may be necessary to ensure high sensitivity for identification of infected cows.

Another important factor in the detection of contagious udder pathogens is the choice of test. Recommendations are only available for diagnosing IMI based on bacterial culture (BC) of two to three consecutive milk samples (Andersen et al., 2010; NMC, 2004). While previous studies investigating the shedding patterns of *Staph. aureus* and *Strep. agalactiae* used BC, polymerase chain reaction (PCR) has generally been used more frequently in recent years. The PCR assay appears to have a higher analytical sensitivity compared to BC (Koskinen et al., 2010; Mahmmod et al., 2013a, 2013b), but may also detect non-viable bacteria (Koskinen et al., 2009), which could lead to lower diagnostic specificity.

Lack of a reference standard presents a challenge in diagnostic test evaluations and investigations of naturally occurring IMI. We would normally base our diagnosis on one or more samples, but we often do not know the true infection status of a quarter based on, for example, a single sample analysed with one chosen test method. Furthermore, as presented by Andersen et al. (2010), the definition of IMI varies with different purposes, and there appears to be some inconsistency in mastitis terminology. Establishing a diagnosis may depend on specific guidelines in combination with the individual's previous experience and own beliefs, thereby further challenging the communication and comparison of efforts to diagnose and control IMI.

The purpose of this study was to improve the diagnostic recommendations for *Staph. aureus* and *Strep. agalactiae* using PCR, BC and somatic cell count (SCC) as diagnostic methods. The objectives were to: 1) describe the observed patterns of diagnostic methods over 21 days in quarters of dairy cows naturally infected with *Staph. aureus* and *Strep. agalactiae*; 2) establish infection types based on a content analysis of expert evaluation of these profiles, and 3) estimate the sensitivity (Se) and specificity (Sp) of each test for each of the infection types established.

Materials and Methods

The objectives were assessed in three steps: firstly, diagnostic test patterns for *Staph. aureus* and *Strep. agalactiae* infections were created by sampling quarters each day for 21 days. Secondly, mastitis experts were asked to describe and group the diagnostic test patterns, and to establish a diagnosis for each group. These groups were subsequently combined to create "infection types".

Thirdly, the diagnostic test results were used to estimate test accuracy in the prediction of established infection types.

Selection of cows and quarters for 21-day profiles

To identify naturally infected quarters with *Staph. aureus* or *Strep. agalactiae*, cows were screened from two Danish Holstein farms with a history of *Strep. agalactiae* IMI established by PCR test on bulk tank milk prior to sampling. On day zero (screening), quarter milk samples were collected aseptically during milking from 589 lactating dairy cows with no signs of clinical mastitis. Initially, cow-level samples pooled from quarter milk samples were tested using PCR (Mastit4, DNA Diagnostic A/S, Risskov, Denmark). Next, the samples positive for *Staph. aureus* (n = 23) or *Strep. agalactiae* (n = 11) were tested using the aforementioned PCR at quarter level, resulting in 43 positive quarters (with one to four quarters/cow). Of these, three quarters were excluded because the cows were treated with antibiotics or culled during the sampling period. This resulted in 16 quarters with *Strep. agalactiae* and 24 quarters with *Staph. aureus* that were investigated over 21 days. PCR test results with a cycle threshold (Ct) of ≤ 37 for *Staph. aureus* and < 40 for *Strep. agalactiae* were deemed positive. The high cut-off for *Strep. agalactiae* was used to ensure inclusion of as many quarters with *Strep. agalactiae* as possible.

Collection of milk samples and laboratory procedures for 21-day profiles

Quarter milk samples were collected daily for a period of 21 days from the quarters identified as positive at screening. The milk samples were collected aseptically after routine preparation of the udder by farm personnel, and before the milking cluster was attached. The teat end was disinfected with cotton pads moistened in 70% ethanol, and two to three squirts of milk were discarded before 30 to 40 mL was collected in a sterile tube. Gloves were changed after sampling each cow. Samples were stored on ice until they arrived at the commercial laboratory (Eurofins, Vejen, Denmark) within a maximum of 4 hours from sampling. At the laboratory, each quarter milk sample was divided into: 1) a bronopol-preserved sample for PCR analysis (Mastit4), 2) a bronopol-preserved sample for SCC analysis (Fossomatic 5000, Foss, Hillerød, Denmark) and 3) a non-preserved sample for BC. The PCR tests and SCC were done by the laboratory personnel, and BC was carried out by the first author. For BC, 0.01 mL of milk was streaked onto an esculin blood agar plate using a sterile disposable loop. The approximate colony forming units (cfu) were counted (up to 300 cfu/0.01 mL) after 24 hours of incubation at 37°C. For all quarters, suspected colonies of *Staph. aureus* and *Strep. agalactiae* were identified and confirmed by Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF; Bruker Biotyper software system, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) twice during the 21 days of sampling. Full genome sequencing and multilocus sequence typing (MLST) were carried out as described by Ronco et al. (2018) for the same isolates.

Presenting the 21-day profiles to mastitis experts

The Ct value, log 10 cfu and log 10 SCC (× 1,000 cells/mL) in quarter milk on each day of the 21-day period were graphed in quarter-profiles. The quarter-profiles were printed on cards with information about the pathogen detected on day zero (screening) and with anonymous quarter ID for use in the expert analysis. It was therefore not possible for the expert to determine whether the quarters were from the same cow or herd.

Respondents and set-up for the expert analysis
Participants at the European Mastitis Research Workers' Conference in Denmark 2017 were asked to interpret and classify the diagnostic test patterns, and to complete a small questionnaire about themselves. The mastitis experts were briefly introduced to the origin of the quarter-profiles and were each provided with an instruction sheet, the 40 quarter-profiles, and eight envelopes; four of which were marked with *Staph. aureus* and four with *Strep. agalactiae*. The experts were asked to make a maximum of four groups (envelopes) for each pathogen and provide a description of each group with the quarter-profiles ("Description"), explain the biology ("Biology"), and finally state a diagnosis in terms of IMI ("Diagnosis"). This open-ended response format is used in questionnaire-based studies in the social sciences (Dillman et al., 2014). The procedure allows participants to provide qualitative accounts (oral, textual, or visual) of, for example, their perceptions of and attitudes to a subject matter. It is a useful technique when the aim is to identify hitherto undocumented human practices. In this case, the technique was used to discern how experts may use temporal patterns in BC, PCR and SCC as diagnostic indicators of *Staph. aureus* and *Strep. agalactiae* infections.

Analysis and coding of responses
Several templates are available for interpreting and coding qualitative responses from human subjects (Miles et al., 2014). In this study, we followed the content analysis approach laid out by Taylor-Powell & Renner (2003). Authors LS and SSN initially read through the qualitative descriptions from respondents and noted all emerging categories. On this basis, ten codes that covered all emerging categories were created and included in a codebook (see Supplemental Table S1). The codes constituted a summary of the "Diagnosis", "Description" and "Biology" written by the respondents. Each code described an infection type (e.g. A4: dynamic infection or A8: transient infection). An additional code (A10) was assigned to inconclusive (because they merely provided a description of the results) or contradictory statements in order to exclude these statements from further analysis. Descriptions of the diagnostic test results without any interpretation would appear in this category. Authors LS, SSN, and APS subsequently coded all responses using the codebook. In cases of disagreement in coding, the text was re-read and discussed among the three coders until consensus was reached about the classification of the text. Prior to disagreements being resolved, overall simple Kappa coefficients were calculated for response agreement among the coders for *Staph. aureus* and *Strep. agalactiae* data independently.

Statistical analysis for estimation of test performance
Dichotomous variables indicating daily infection status were constructed for each test (0 = negative; 1 = positive) based on daily BC, PCR and SCC test results. BC was considered positive at a detection limit corresponding to 100 cfu/mL. The PCR test was considered positive at Ct ≤ 37 for both *Staph. aureus* and *Strep. agalactiae*, as suggested by the manufacturer. SCC was dichotomised at the 100,000 cells/mL cut-off, as suggested for quarter milk samples (IDF, 2013).

Subsequently, a dichotomous expert-based infection variable was created to use as a reference to evaluate the daily test results. Every day was assigned a separate value for this infection variable for each quarter and for each of the experts. If the quarter was coded A1 or A2 for any given expert, the quarter was considered not-infected (Table 1) and the infection variable was 0 for all 21 days. Quarters that were coded A3, A4 or A5 were considered to be infected throughout the whole period, and the infection variable was therefore set to 1 for all 21 days. Quarters that were coded A6, A7, A8 or A9 were considered to be infected for fewer than 21 days during the period. In these cases, the value assigned to the infection variable (either 0 or 1) was determined by the first author for each of the 21 days, depending on the pattern in the daily test results and the infection type code.

Table 1. Number of envelopes from expert analysis assigned to each of the ten codes from the codebook, and the overall infection status based on the codes

Code (Infection type)	*Staph. aureus* No. of envelopes (%)	*Strep. agalactiae* No. of envelopes (%)	Conclusion
A1: True negative	14 (12.0%)	16 (14.3%)	Not infected
A2: False positive	12 (10.3%)	11 (9.8%)	
A3: True positive (persistent)	30 (25.6%)	22 (19.6%)	Infected
A4: True positive (dynamic)	30 (25.6%)	9 (8.0%)	
A5: False negative (persistent)	6 (5.1%)	16 (14.3%)	
A6: True positive (new infection)	0 (0%)	8 (7.1%)	Infected, but only for some of the period
A7: False negative (new infection)	0 (0%)	1 (0.9%)	
A8: Transient infection	11 (9.4%)	4 (3.6%)	
A9: Resolving infection	1 (0.9%)	4 (3.6%)	
A10: Not Applicable	13 (11.1%)	21 (18.8%)	-
Total	117	112	-

Generalised logistic regression models were subsequently used to estimate the Se and Sp of BC, PCR and SCC for the different infection types of *Staph. aureus* and *Strep. agalactiae*. In these models, the expert-based infection variable was used as the outcome. The daily test result of BC, PCR or SCC was included as a fixed effect together with the different infection types (using the codes from Table 1). Quarter ID and expert were included as random effects. The full model was reduced using backward elimination based on the P-value ($P < 0.05$) and model convergence. Assessment of under-dispersion was used to evaluate the model fit. Under-dispersion existed in many cases because there were limited variation in the results. Due to lack of model fit when including the full dataset, a random sample of n test-days per expert per quarter was assessed until under-dispersion was deemed not to occur. With *Strep. agalactiae* PCR and BC test results as the outcome, the model was fit using 11 test-days per expert per quarter and all test-days, respectively. For the *Strep. agalactiae* SCC and *Staph. aureus* models, model fit was only achieved with 1 randomly selected test-day per expert per quarter, and the random statements were therefore excluded. Overall infection Se and Sp, in addition to Se and Sp of the different infection types were calculated based on the output from each of the models and according to model fit and data availability. In some cases, variables were excluded from the model or there were no observations to calculate a given estimate, so some estimates were not applicable (e.g. Sp could only be estimated for periods during which the cows were deemed to be non-infected).

The random data sampling, Kappa calculation and logistic analyses were carried out using the Surveyselect, Proc freq and the Glimmix procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC).

Results

The 21-day profiles

The cows (n = 31) included in the study were in first to fifth lactation (median = second) and from 12 to 505 days in milk (DIM; median = 176) at screening. Of the 40 quarters studied (24 with *Staph. aureus* and 16 with *Strep. agalactiae*), four were dried off on day 20, resulting in 836 quarter-days available. The SCC and PCR analyses failed in ten and seven samples, respectively, and these were consequently not plotted. Examples of quarter-profiles are shown in Figure 1,

where variation can be observed in the patterns of both *Staph. aureus* and *Strep. agalactiae*. All 40 quarter-profiles are shown in Supplemental Figure S2.

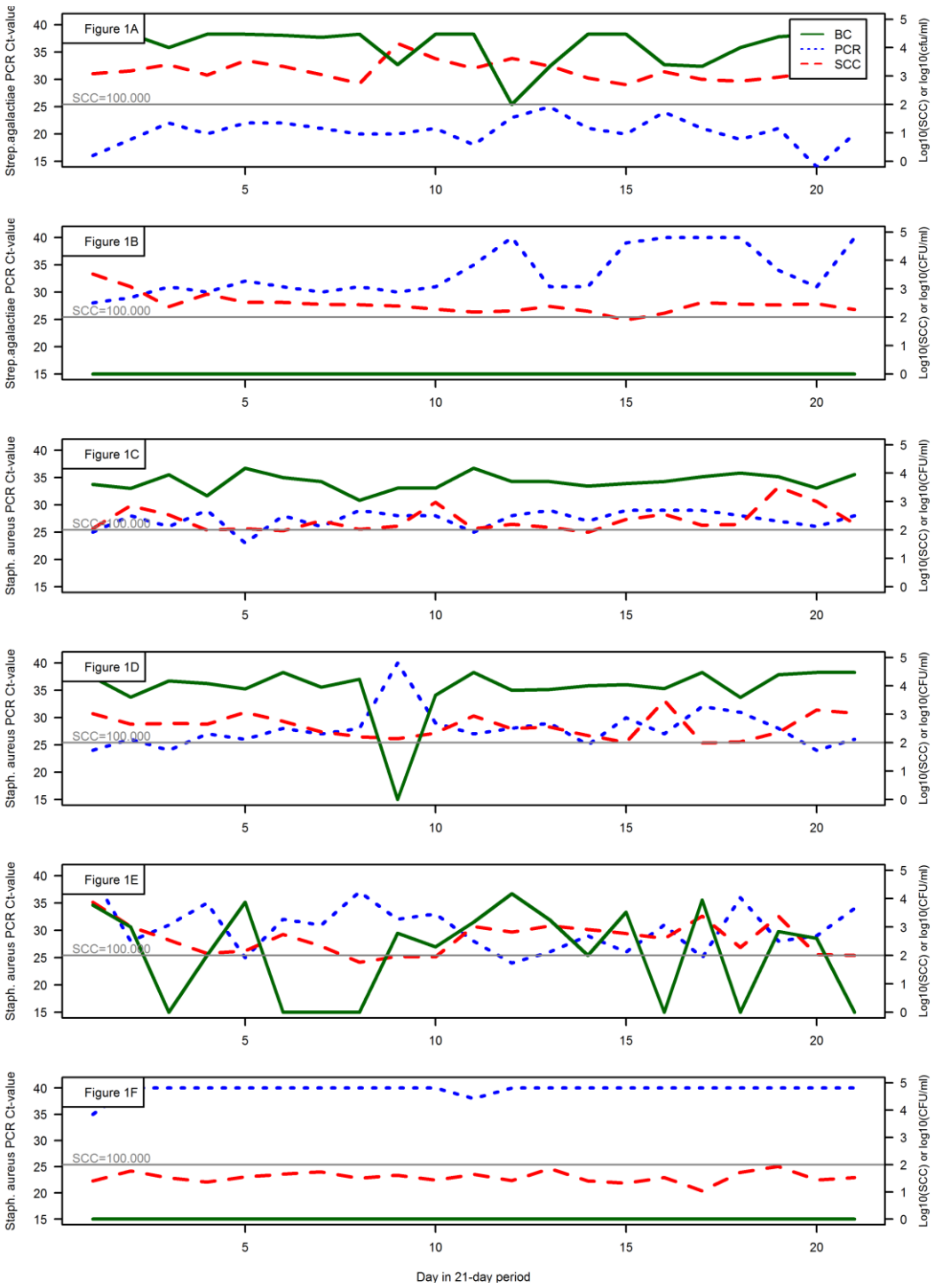

Figure 1. Examples of quarters with *Streptococcus agalactiae* (A and B) and *Staphylococcus aureus* (C, D, E, F) investigated over 21 days with daily bacterial culture (BC; solid line), polymerase chain reaction (PCR; dotted line) and somatic cell count (SCC; dashed line) test results.

Expert analysis
Statements from 30 experts were included. There was an equal distribution of men and women, and 80% had more than 2 years of experience in working with mastitis. Almost half of the experts stated that they spent more than 50% of their annual working time on mastitis-related work. Their main occupational areas included: epidemiology, microbiology, diagnostics, udder health consultancy and statistical modelling. A number of experts also worked in immunology and some did clinical work.

All descriptions, statements explaining the biology, and diagnoses provided by the experts are given in Supplemental Table S3. The coding results from each coder were compared for each pathogen, resulting in overall simple Kappa values of 0.82 [0.78; 0.86] and 0.77 [0.72; 0.81] for the *Staph. aureus* and *Strep. agalactiae* data, respectively. The distribution of final codes assigned at envelope level after disagreement among coders was resolved is shown in Table 1. For *Strep. agalactiae*, 112 envelopes were coded and 21 of those were assigned Code A10 and therefore excluded from further analysis. One expert categorised into two groups (envelopes), six experts categorised into three groups, and the remaining 23 experts (77%) used the maximum number of groups allowed (4). For *Staph. aureus*, three experts categorised into three groups and the other 27 experts (90%) used all four groups. Of 117 envelopes, 13 were assigned the Code A10 and were excluded from further analysis. Almost all experts described a non-infected group (Codes A1 and A2) for both pathogens. A persistent infection group (Codes A3 and A5) was the most frequent among the *Strep. agalactiae* profiles, whereas a persistent (Code A3) and a dynamic infection (Code A4) were the most frequent groups among the *Staph. aureus* profiles (Table 1). New infections (Codes A6 and A7) were only described for *Strep. agalactiae*.

Experts' diagnoses using 21-day profiles
For *Staph. aureus*, 17 out of 24 quarters (71%) were mainly coded persistently infected (Code A3), and in some cases dynamic (Code A4) or persistent with false-negative test results (Code A5). Examples of quarters in this group are shown in Figures 1C and 1D. The same was true for 56% (9 out of 16) of the quarters with *Strep. agalactiae* (example in Figure 1A). Two quarters were mainly identified as non-infected for *Strep. agalactiae* and four quarters were mainly identified as non-infected for *Staph. aureus*. A quarter from the non-infected group of *Staph. aureus* is shown in Figure 1F. For *Strep. agalactiae*, five quarters were inconsistently grouped by the experts into either non-infected or infected (example in Figure 1B), including resolving infection (A9) and new infection (A6 or A7). For *Staph. aureus*, three quarters were mainly coded as a dynamic infection (A4, example in Figure 1E). Quarters with *Staph. aureus* were primarily sequence type (ST) 50, but ST 72, ST 45, ST 133, ST 151, and ST 504 were also represented. The quarters with dynamic *Staph. aureus* infections were ST 45, ST 71, and ST 133. For quarters with *Strep. agalactiae*, ST 8 represented quarters from one herd and ST 626 represented quarters from the other herd. The same ST were detected twice for all quarters over the 21 days.

Test performance
The test performance of BC, PCR and SCC calculated based on experts' diagnoses (infection types) and model output is shown in Table 2. The Se estimates were generally high, for example, the Se of BC for *Strep. agalactiae* was 100% for infections and new infections. The only exception to this was the Se of SCC for detecting *Strep. agalactiae* infections (34.3%), as well as transient infections with both *Strep. agalactiae* (9.1%) and *Staph. aureus* (43.5%). The calculated Sp for overall infection was generally low, except for the Sp of BC (99%) and PCR (97.7%) in detecting *Strep. agalactiae*.

Table 2. Estimates of sensitivity (Se) and specificity (Sp) for bacterial culture (BC), polymerase chain reaction (PCR), and somatic cell count (SCC) from model output for different infection types using experts' diagnoses as reference. In some cases, a low number of observations were used to avoid under-dispersion and obtain model fit, and it was therefore not possible to estimate the Se and Sp for some infection types (NA = not applicable)

Infection type	Test parameter	*Staph. aureus*		*Strep. agalactiae*	
		%	95 % CI	%	95 % CI
Overall infection	Se_{BC}	95.9	(93.7; 97.3)	100	(83.5; 100)
	Sp_{BC}	74.5	(65.7; 81.7)	99.0	(72.8; 100)
	Se_{PCR}	99.5	(98.3; 99.8)	99.9	(99.6; 100)
	Sp_{PCR}	66.0	(57.2; 73.8)	97.7	(62.1; 99.9)
	Se_{SCC}	96.1	(94.0; 97.5)	34.3	(26.4; 43.3)
	Sp_{SCC}	43.7	(36.2; 51.5)	65.7	(56.7; 73.7)
New infection	Se_{BC}	NA	NA	100	(99.7; 100)
	Sp_{BC}	NA	NA	13.6	(4.4; 35.0)
	Se_{PCR}	NA	NA	100	(97.8; 100)
	Sp_{PCR}	NA	NA	NA	NA
	Se_{SCC}	NA	NA	NA	NA
	Sp_{SCC}	NA	NA	NA	NA
Transient infection	Se_{BC}	96.5	(77.4; 99.5)	NA	NA
	Sp_{BC}	95.0	(76.3; 99.1)	NA	NA
	Se_{PCR}	94.3	(77.4; 99.0)	99.8	(96.3; 100)
	Sp_{PCR}	91.0	(72.4; 97.5)	99.0	(55.5; 100)
	Se_{SCC}	43.5	(20.0; 70.3)	9.1	(2.5; 27.6)
	Sp_{SCC}	96.2	(88.3; 98.8)	91.0	(72.4; 97.5)
Resolving infection	Se_{BC}	NA	NA	NA	NA
	Sp_{BC}	NA	NA	NA	NA
	Se_{PCR}	NA	NA	85.8	(28.0; 99.0)
	Sp_{PCR}	NA	NA	96.9	(67.5; 99.8)
	Se_{SCC}	NA	NA	NA	NA
	Sp_{SCC}	NA	NA	75.0	(23.7; 96.6)

Discussion

The current study included three steps combining quantitative and qualitative methods in order to address the three objectives. To our knowledge, this is the first study to evaluate test accuracy based on 21-day profiles using mastitis experts to set the reference standards.

21-day profiles

Some of the quarters showed a consistent – and some a more varying – pattern of *Staph. aureus* or *Strep. agalactiae* infection. For *Staph. aureus*, we found that the diagnostic test patterns were mainly consistent and in line with reports by Walker et al. (2011), who investigated seven naturally infected cows for 21 days, evaluating SCC and BC. However, we also observed quarters with more variation, especially in BC results (example in Figure 1E), for which repeated sampling would be necessary to assess the diagnosis. These dynamic patterns were in line with the findings reported by Sears et al. (1990), who mainly investigated experimentally infected quarters (n = 19), as well as naturally infected quarters (n = 4) over 65 days, and found a low- and a high-shedding cycle using a mean cfu > 1,000 cfu/mL as a cut off. The low-shedding cycle could correspond to the more inconsistent patterns that we found using BC. In contrast to the current study, Studer et al. (2008) found that the shedding patterns obtained using PCR and BC were

synchronous for *Staph. aureus*. Furthermore, they observed one to four major shedding peaks in all 11 naturally infected quarters investigated over 14 days, whereas we mainly observed more consistent shedding patterns in the current study.

Thieme and Haasmann (1978) reported highly variable shedding of *Strep. agalactiae* at quarter level from ten naturally infected cows classified as low shedders (unknown criteria). Their detection of *Strep. agalactiae* was only based on BC. In contrast, we were either able to culture *Strep. agalactiae* over the period of 21 days (Figure 1A) or not at all, despite a positive screening and an occasionally positive PCR test indicating that bacterial DNA was present (Figure 1B). These patterns appeared difficult for the experts to interpret consistently, as they were assigned to almost all different infection groups (Supplemental Figure S2). However, the disagreement between BC and PCR in those profiles was in line with what has been observed for experimental Staphylococcus spp.-infected quarters in which PCR and SCC results were positive for weeks after the quarters were recorded as spontaneously cured according to the BC (Hiitiö et al., 2018).

The method used to select quarters to study is likely to cause the difference in results among studies. We selected our quarters based on a single PCR-positive sample with a high Ct as a cut-off especially for *Strep. agalactiae* (at cow and quarter level), whereas the other studies selected infected quarters based on repeated positive BC samples. A consequence of using PCR on a single milk sample with such a high Ct as a cut-off is that we also investigated apparently negative quarters for both *Staph. aureus* and *Strep. agalactiae*, despite the positive PCR sample on day zero (screening). This indicates that treatment of infected quarters should not rely on a single positive PCR sample. However, this selection of "weak" infections provided another insight into shedding or diagnostic test patterns of *Staph. aureus* and *Strep. agalactiae* infection.

There were also differences in test methods among the studies, with a different PCR method used by Studer et al. (2008), as well as differences between the BC methods used (mL of milk plated and agar used). We used the culture method recommend by NMC (2004) – a method also used by the majority of Danish herd veterinarians who culture milk samples. Furthermore, the applied PCR assay and SCC test are used in routine diagnostics in Denmark. Consequently, the setup resembles clinical practice.

Expert analysis and diagnoses of 21-day profiles

From the distribution of infection types (Table 1), it appears that *Staph. aureus* was more often assigned to a dynamic infection pattern compared to *Strep. agalactiae*, whereas only quarters with *Strep. agalactiae* were assigned to new infections. These differences could be caused by pathogen differences, our selection of quarters, or the experts' interpretation of the profiles being pathogen-dependent. However, based on the ST for the different quarters, it was hard to determine whether the ST of the pathogen was associated with the shedding pattern due to the low number of quarters investigated. For *Strep. agalactiae*, a single strain was found in each herd, which is in line with previous studies (Jørgensen et al., 2016; Zadoks et al., 2011). For *Staph. aureus*, multiple strains were present among the quarters studied. ST 45 and ST 133 were found in quarters diagnosed as persistent as well as dynamic infections. As such, only ST 71 was found in dynamic infections and not in persistent infections.

A number of envelopes were excluded mainly due to inconclusive statements from the experts. Some statements were contradictory, perhaps due to a confusing or inconsistent use of mastitis terminology. Furthermore, the quarter profiles mainly assigned Code A10 were quarters with *Strep. agalactiae* such as no. 02, 18 and 22 in Supplementary Figure S2, probably because these quarters presented unexpected patterns and disagreement between diagnostic test results. We would normally base our diagnosis on one or more samples (NMC, 2004; Andersen et al., 2010),

and we do not know if the individual result is true or false, but we rely on knowledge of the overall accuracy of the test used, along with the prevalence to assess the positive or negative predictive value. Different terminology is required when assessing patterns, and the experts may not have a common reference terminology. Furthermore, as the experts were presented patterns of three different tests (BC, PCR and SCC) that did not always agree, much weight was put on the individual expert's preference and interpretation of the different tests.

Only four groups were allowed for each pathogen, meaning that some patterns could be assigned to the same diagnosis without completely fitting the diagnosis group. Furthermore, the codebook was dependent on the authors' interpretation, and it would be relevant to validate the codes by presenting them to the same experts, or to have a new group of experts allocate profiles within the frame of the developed codebook.

Test performance

The Se estimates for *Staph. aureus* detection in an overall infection were high (Table 2). Our estimate of Se for BC (95.9%) was comparable with the Se of BC (97.5%) reported by Walker et al. (2011). In contrast, Sears et al. (1990) reported a lower overall Se (74.5%) due to low-shedding quarters with a mean cfu < 1,000 cfu/mL, and Studer et al. (2008) reported an overall Se for BC of 79.9%, using 0.01 mL of milk for plating. Furthermore, Studer et al. (2008) reported an overall Sp of 100% for BC, which is significantly higher than that obtained in the current study (74.5%). However, our quarters were initially PCR positive and therefore potentially subject to spectrum bias, but also more likely to start out as false-positive subjects. It should also be noted that the data used for estimating Sp in the current study were sparse because we aimed to include only infected quarters, but ultimately included quarters that appeared to be non-infected.

The overall infection estimates of Sp using BC were generally comparable with those estimated in studies using a latent class approach (Mahmmod et al., 2013a; b; Svennesen et al., 2018). In contrast, the Se estimates of BC for both *Staph. aureus* and *Strep. agalactiae* were high compared to previous reports of around 50% (Mahmmod et al., 2013a; b; Holmøy et al., 2018; Svennesen et al., 2018), which could be due to the spectrum bias mentioned above.

Studer et al. (2008) reported a PCR Se and Sp for *Staph. aureus* of 99.4% and 97.1%, respectively. In the current study, the Se was at the same level (99.5%), yet the Sp was remarkably lower (66%). In addition, the Sp of PCR in the current study was also low when compared to the Sp of PCR estimated in studies using a latent class approach (Mahmmod et al., 2013b; Svennesen et al., 2018). The Se of SCC at quarter level (using a cut-off of 100,000 cells/mL) for detection of *Staph. aureus* was high, but this was the test with the lowest Sp (43.7%). The Se (96.1%) was higher than previously reported (83.3%) for infection with different pathogens (including *Staph. aureus*) when results of a quarter-level SCC with the same threshold were evaluated against BC results (Schepers et al., 1997). However, the Sp in the current study was lower compared to a previously reported Sp of 80.5%.

Both the Se and Sp of SCC were low for *Strep. agalactiae*. In contrast, the Se and Sp of both BC and PCR were high (overall infection estimates). This indicates that positive BC and PCR test results for *Strep. agalactiae* were more important than SCC results when experts diagnosed a quarter as infected. Previously, Thieme and Haasmann (1978) investigated low-shedding *Strep. agalactiae* cows with BC, obtaining an Se of only 26%. However, Dinsmore et al. (1991) reported a BC Se of 98.8% based on three milk samples taken over 2 weeks, where an infected case was defined as two positive samples. The main differences between those studies may have been the selection of infected quarters and the number of samples used to asses Se and Sp.

The estimates indicate which elements the experts deem important for both new and resolving infections (Table 2). For new infections the Se was high and the Sp was low, meaning that emphasis was put on detection of new infections, even if they were false-positives. In contrast, the Sp was high for resolving infections, whereas the Se would probably be lower if we had more data available. It should, however, be noted that the infection variables for quarters in Code A6 – A9 (infected for fewer than 21 days throughout the period) were assigned on a daily basis by the first author, which may lead to uncertainty about these estimates.

For transient infections, it was possible to obtain relatively high Se and Sp estimates when we had the opportunity to look at a longer timescale of shedding to assess whether the infection was permanent or transient. Furthermore, the Se of SCC in transient infections was low, indicating that SCC was not appropriate in assessing this type of infection, perhaps due to a longer response time for a short-lived infection (Hiitiö et al., 2018). However, the SCC results should be interpreted with caution as other pathogens were sometimes present in the investigated quarters and thereby could cause an increased SCC (data not shown). Furthermore, we used a relatively low SCC cut-off, which could explain the low Sp of SCC for overall infections (Schepers et al., 1997).

In relation to treatment and culling in the control of contagious udder pathogens, it might be important to assess whether infections are persistent (chronic) or transient, as the best (most cost-effective) strategy may depend on the infection type (Gussmann, 2018). Most importantly, the profiles of new, transient, or resolving infections can explain why the estimates of overall infection do not often reach values of 100%.

An approach using experts to set the true infection status, which is often needed in mastitis research, was previously used by Andersen et al. (2010), who used expert opinion to create rules for IMI diagnosis or classification based on simulated combinations of three BC test results. The use of experts makes it possible to add another level of interpretation to our classification of investigated quarters. However, the number of included quarters was low, and greater variation would have been beneficial for modelling in order to include more observations per expert and quarter.

Conclusion

We analysed statements from mastitis experts to set the reference standard of infection type for 21-day diagnostic test patterns of quarters with *Staph. aureus* or *Strep. agalactiae*. Experts mainly identified consistent patterns for *Staph. aureus*, although a small number of patterns were more dynamic. *Strep. agalactiae* quarter-patterns were either consistent or hard to diagnose, as experts did not agree on the diagnosis of these quarters. Furthermore, we found disagreement between the BC and PCR test results for some quarters. Based on the diagnostic test patterns, we suggest that a diagnosis should not rely on a single sample or test result alone.

Using the experts' diagnoses to estimate the Se and Sp for an overall infection resulted in high BC and PCR Se, for example, the Se of BC for *Strep. agalactiae* was 100% [83.5; 100]. The Sp of BC and PCR for *Strep. agalactiae* were 99% [72.8; 100] and 97.7% [62.1; 99.9], respectively, whereas the Se of SCC for detecting *Strep. agalactiae* was only 34.3% [26.4; 43.3]. This indicates that positive BC and PCR test results for *Strep. agalactiae* were more important than SCC results when experts were diagnosing a quarter as infected. In contrast, the Se of SCC in detecting *Staph. aureus* was high (96.1% [94.0; 97.5]), but the Sp estimates of all tests were lower, i.e. 74.5% [65.7; 81.7], 66% [57.2; 73.8] and 43.7% [36.2; 51.5] for BC, PCR and SCC, respectively.

We conclude that both PCR and BC are highly sensitive for the detection of persistent and new infections as defined by the experts, although the Se was not always 100%. In addition, the

accepted lower Sp suggests that experts put less emphasis on a false-positive result. We recommend that efforts are made to develop consistent terminology to characterise IMI over time so the course of infection can be taken into account when diagnosing IMI.

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Supplemental Table S1

Codebook and code system

Diagnoses: The diagnosis codes were based on the overall description and conclusion from the expert (including text box: “Label”, “Description” and “Biology”). One code should be given to each envelope (row in excel, Supplemental Table S3).

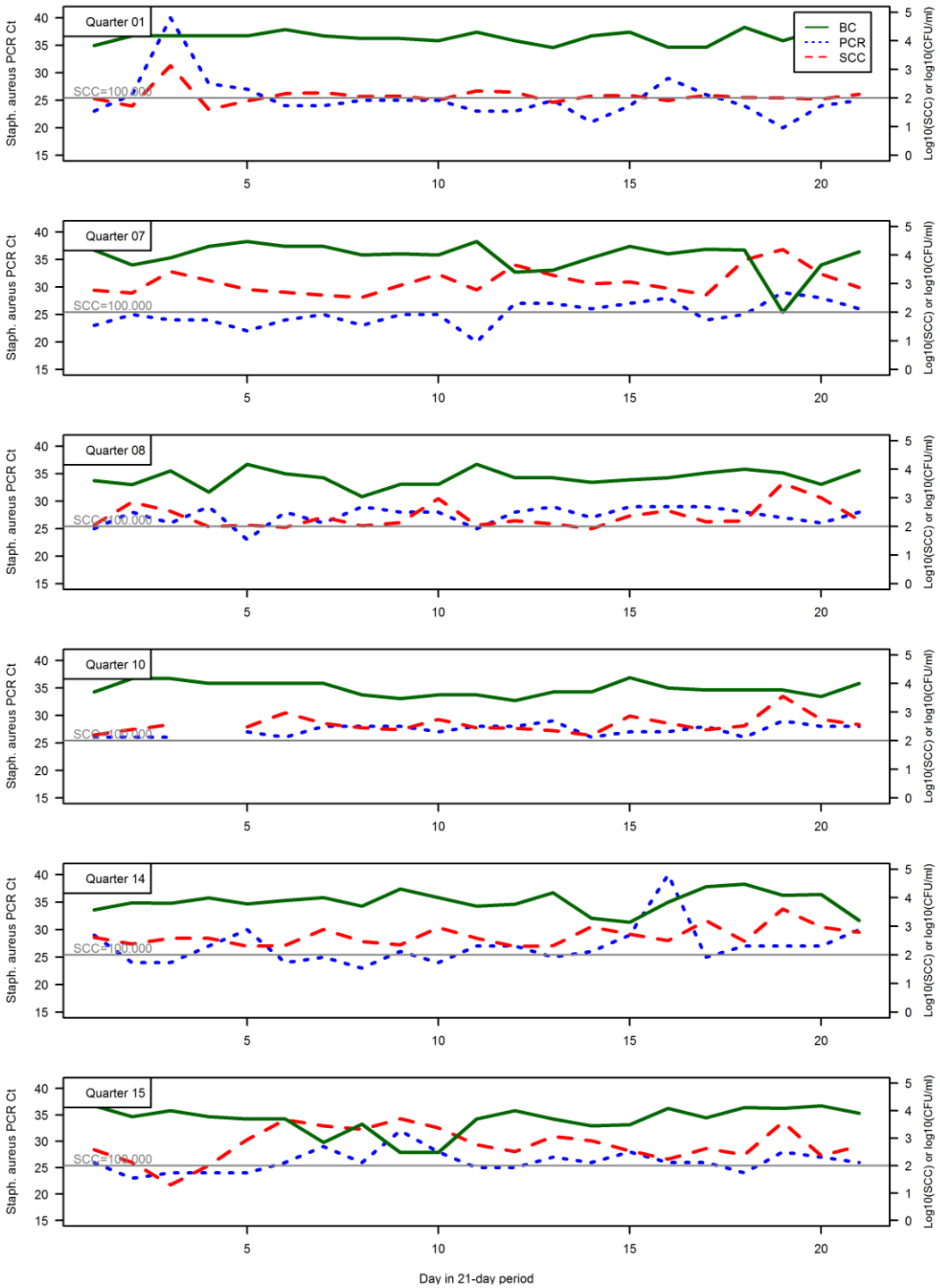
Code	Use	Key-words
A1. True negative	This code was used when the expert´s conclusion included only “healthy”, “negative” or similar terms. Eventually described as all tests negative throughout the period. This code does not include “healthy” quarters for which single positive test results were mentioned (see A2. False-positive).	Healthy, negative, non-infected, free of infection
A2. False positive	This code was used when some positive test results were described additionally to the expert´s conclusion being “healthy”, “negative” or similar, meaning that the positive test results were by the expert understood as false positive. This code also includes false positives mentioned as e.g. “test error”, “contamination” and “carry-over”.	Healthy, negative, non-infected, free of infection – expert describes one or several positive test results as not important, including contamination, false positive, wrong, carry over, error, other source of infection (teat skin, teat canal)
A3. True positive (Persistent)	This code was used when a quarter was described as infected, with a continuous shedding pattern. This code should not include false negative test results which are covered by A5. False negative persistent.	Steady, high shedding, always shed, chronic, persistent
A4. True positive (Dynamic)	This code was used when a dynamic or fluctuating pattern was described for a quarter that was considered infected. In this code the expert believes that this is the biological shedding pattern and not related to test properties.	Low shedding, low infection, fluctuating, varying, dynamic, uncontrolled, mostly positive, more or less consistently
A5. False negative (FN Persistent)	This code was used when a quarter was described as infected, with a continuous shedding pattern, but also including false negative test results at a specific point in time or disagreement between tests at one or a few time-points. In this code the negative test results are related to test properties.	CFU detection limit to low, occasional no growth
A6. True positive (New)	This code was used when a new or acute infection was described, meaning that the quarter changed from healthy to infected during the period. Acute infection with positive test results through the whole period should not be included here but in A3. True positive persistent.	New infection, acute infection, first negative – then positive, cow infected early in period

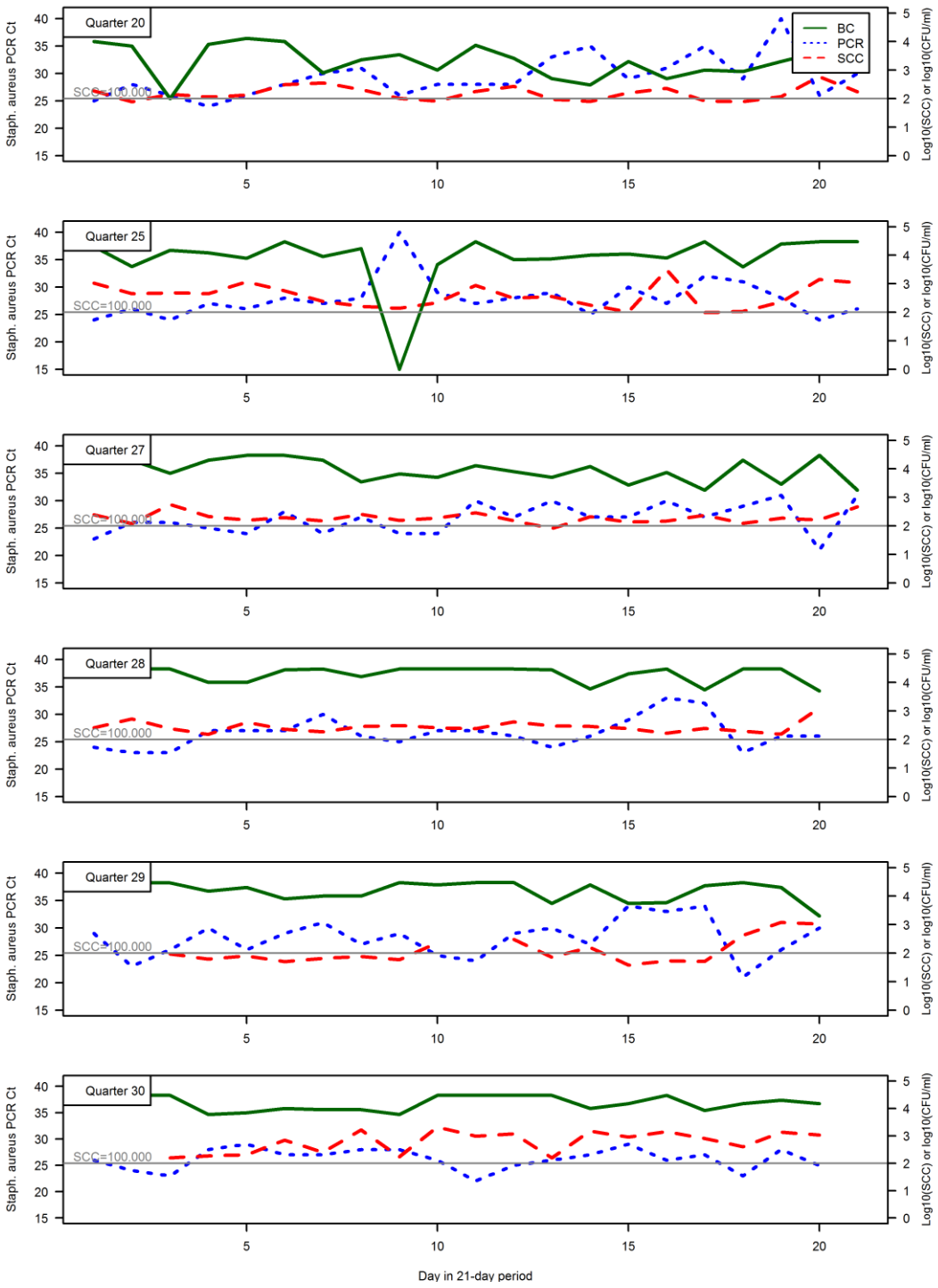
A7. False negative (FN new)	This code was used when one or several false negative test results were described for a new or acute infection at a specific point in time after point of infection.	New or acute infection with false negative
A8. Transient infection	This code was used when a transient infection was described, meaning that the infection started and ended within the period. Infections only starting should be coded A6. True positive new. If the infection was only ending within the period, see A9. Resolving infection.	Peak, moment of positives, short-lasting infection
A9. Resolving infection	This code was used when a recovery was described, understood by end of infection within the period.	Recovering, immune system gets rid of infection, was infected, cure(d)
A10. Not applicable	This code was used when the answer did not fit the question or if the text box was empty. Furthermore, if the expert was inconclusive (merely describing test-results) or if the statements were contradictory.	Unknown, inconclusive, can't explain, unusual, no idea,

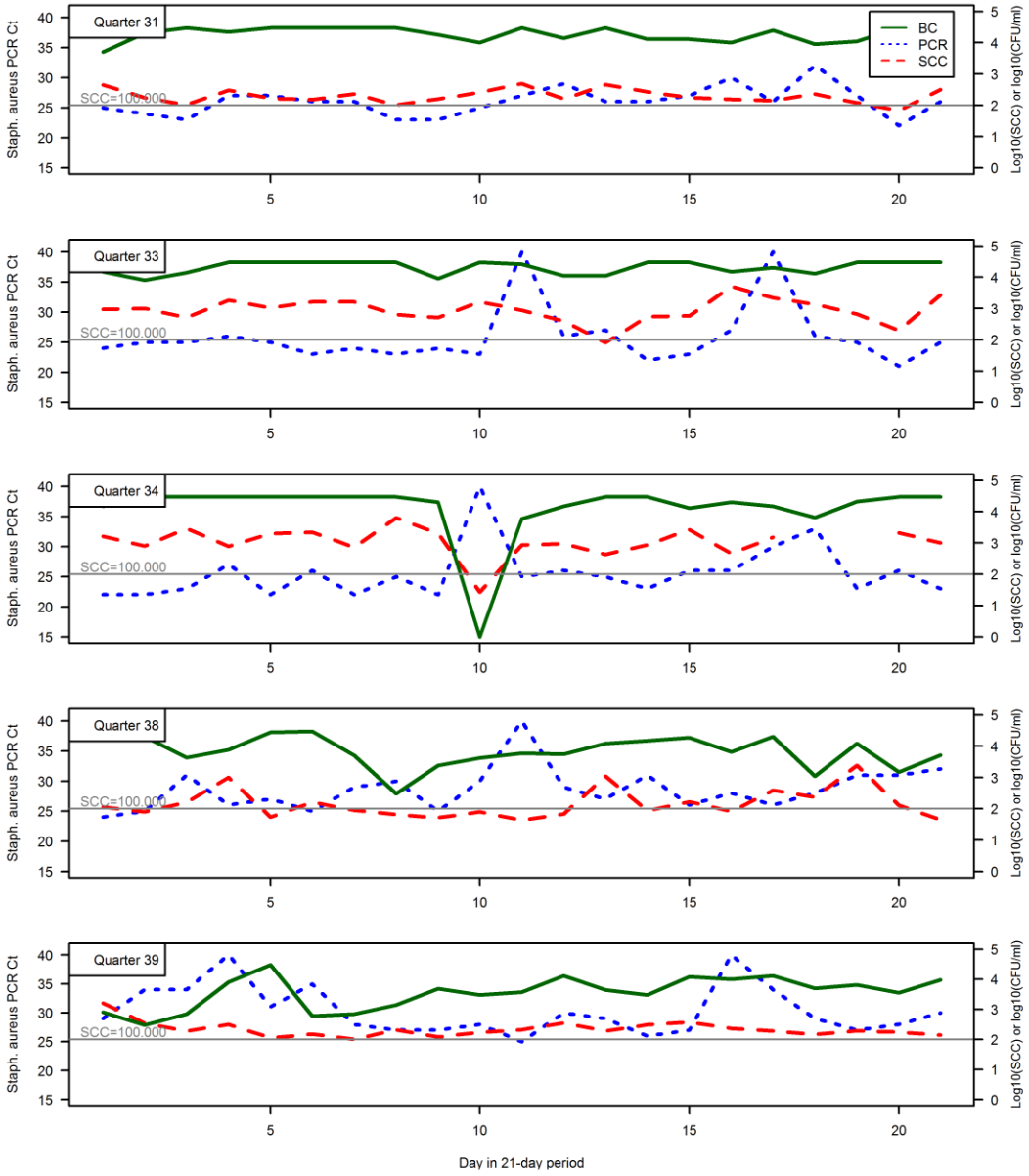
Supplemental Figure S2

Profiles of 40 quarters followed for 21 days with bacterial culture (BC), PCR and SCC

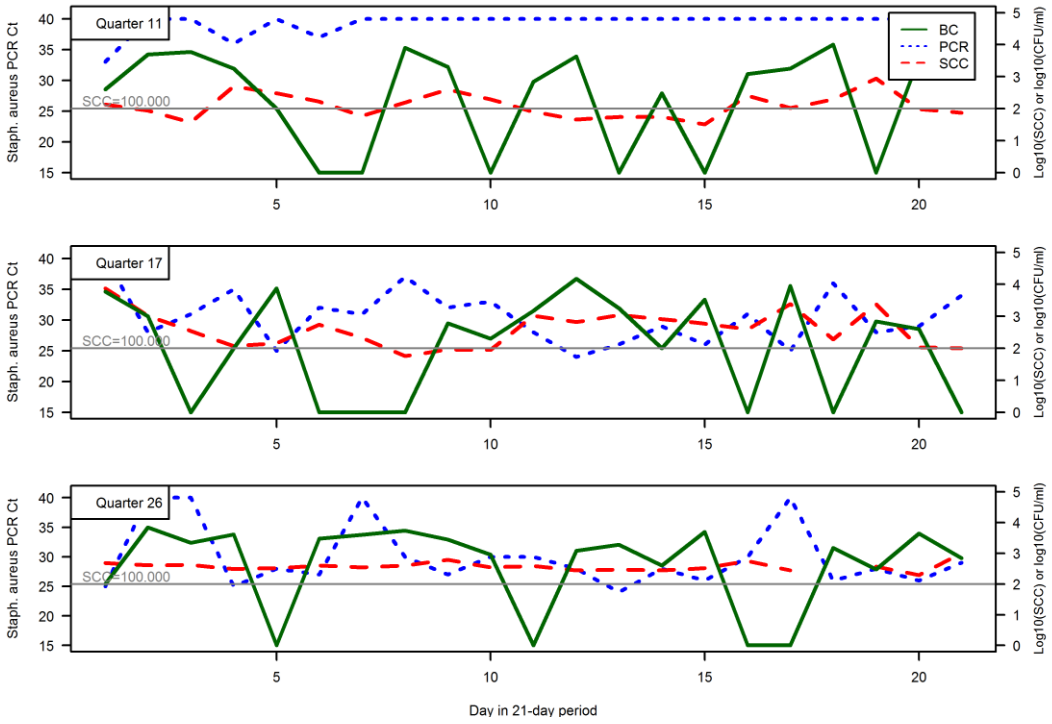
The following 17 out of 24 *Staphylococcus aureus* quarters were diagnosed as infected, mainly persistent infected, and in some cases dynamic or persistent with false negative test results.



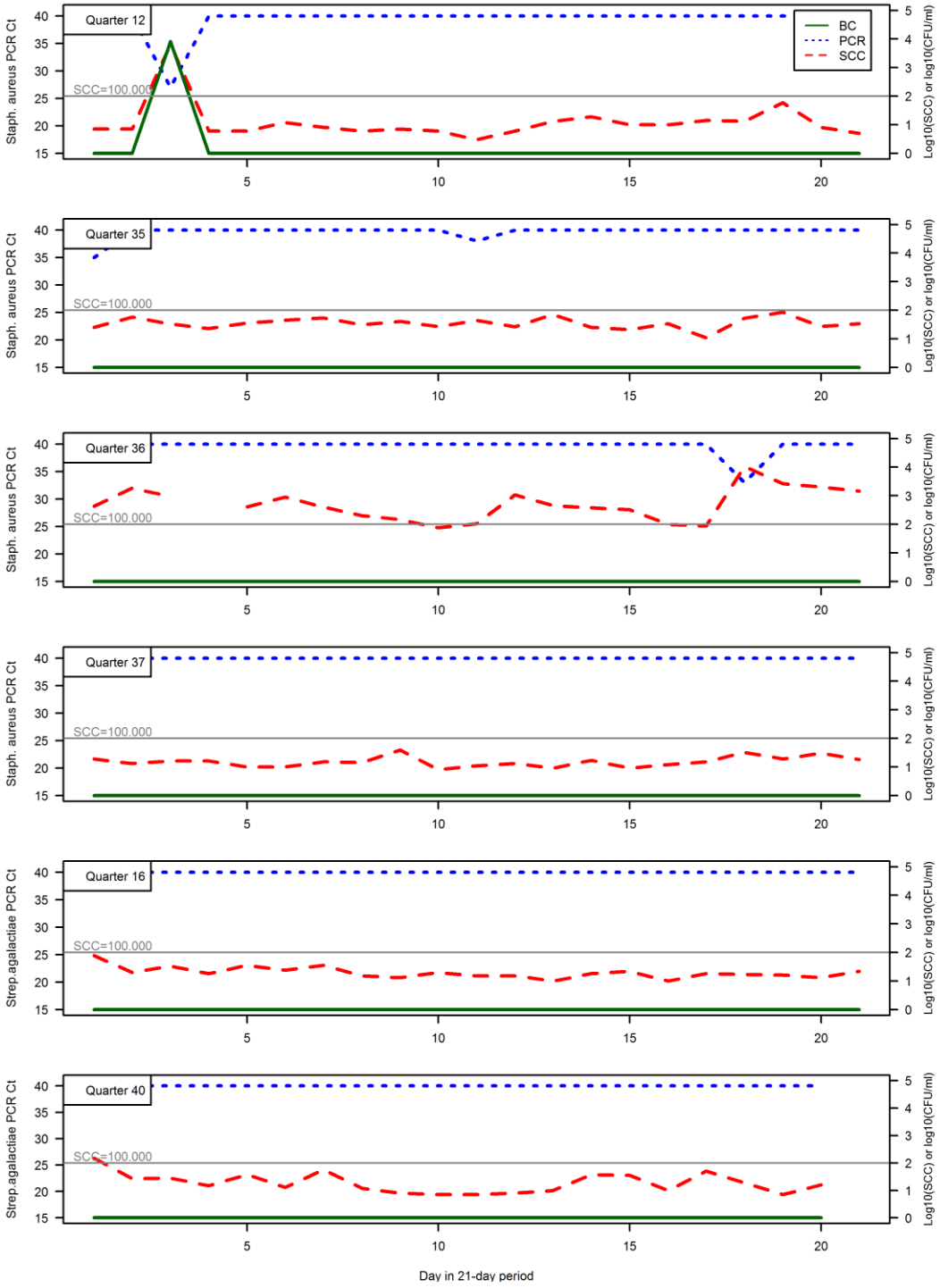

Quarter 20
Quarter 25
Quarter 27
Quarter 28
Quarter 29
Quarter 30
BC
PCR
SCC
SCC=100.000
Staph. aureus PCR Ct
Log10(SCC) or log10(CFU/ml)
Day in 21-day period


Quarter 31
Quarter 33
Quarter 34
Quarter 38
Quarter 39
BC
PCR
SCC
SCC=100.000
Staph. aureus PCR Ct
Log10(SCC) or log10(CFU/ml)
Day in 21-day period

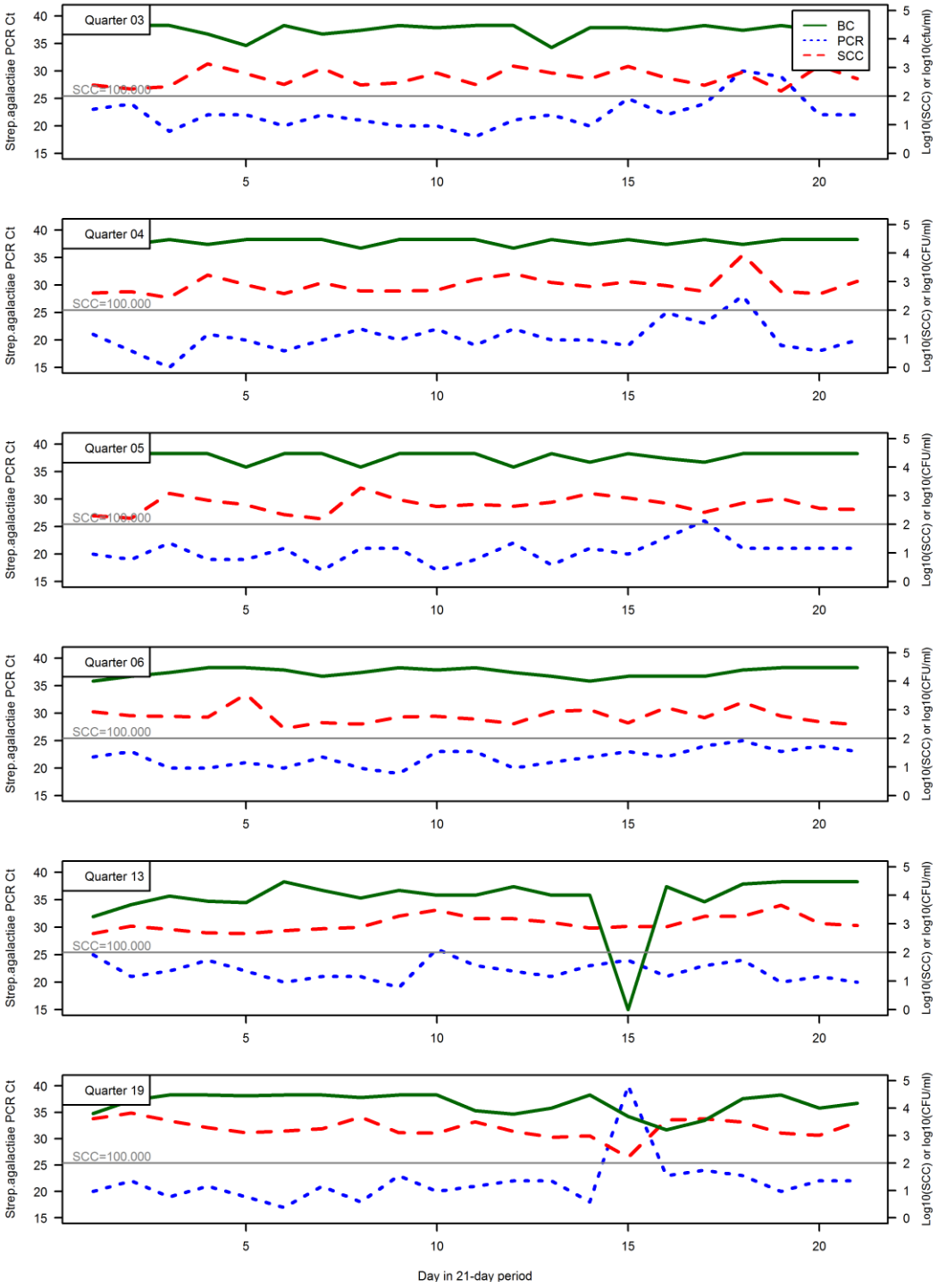
The following three *Staphylococcus aureus* quarters were mainly diagnosed as dynamic infections.

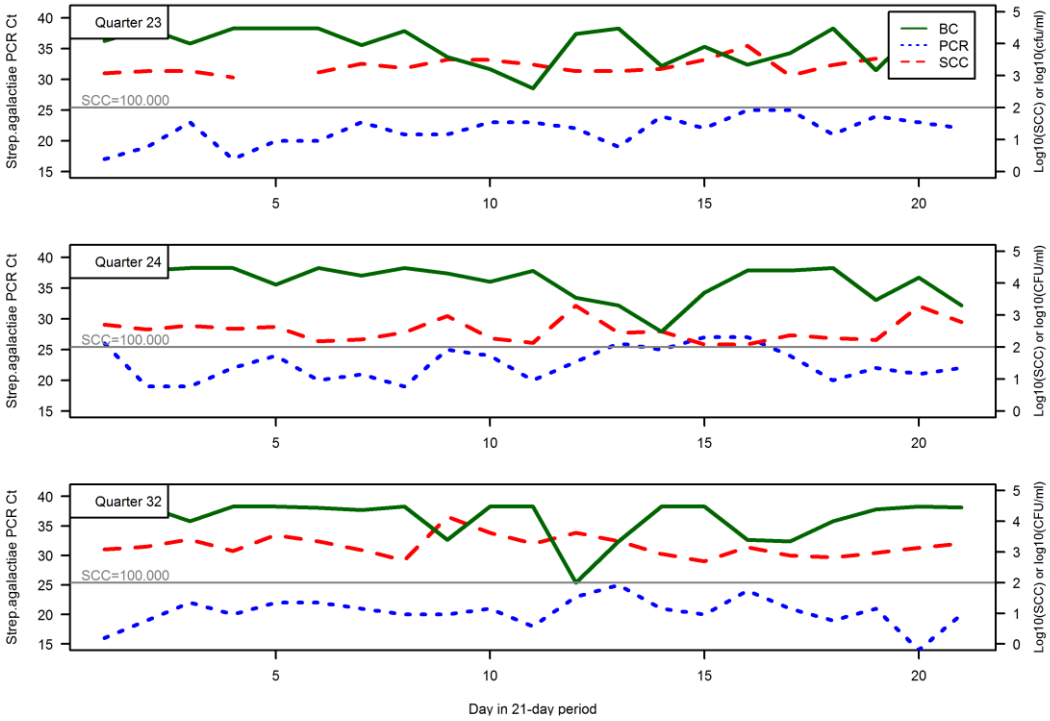


The following 6 quarters were mainly diagnosed as non-infected, including four *Staphylococcus aureus* quarters and two *Streptococcus agalactiae* quarters.

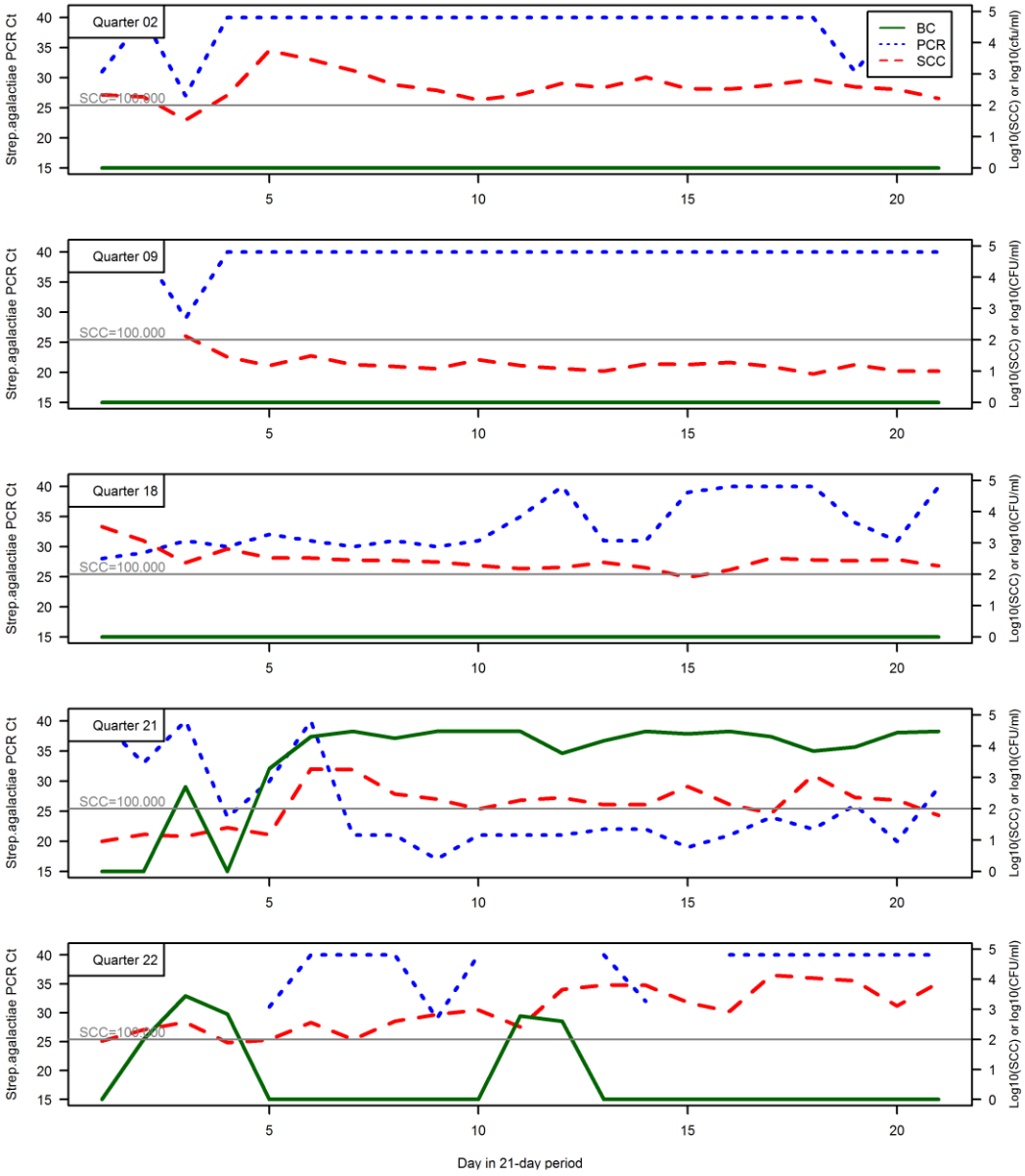


The following 9 out of 16 *Streptococcus agalactiae* quarters were mainly diagnosed as infected; persistent, dynamic or persistent with false negative test results.




Quarter 23
Quarter 24
Quarter 32
BC
PCR
SCC
SCC=100.000
Strep.agalactiae PCR Ct
Log10(SCC) or log10(cfu/ml)
Log10(SCC) or log10(CFU/ml)
Day in 21-day period

Five *Streptococcus agalactiae* quarters coded everything from infected to non-infected; including recovering and new-infections.



Staphylococcus aureus					
Expert ID	**Envelope #**	**Label (Expert)**	**Description (Expert)**	**Biology (Expert)**	**Code (authors)**
1	1	Chronic infections/subclinical mastitis	Chronic shedder always positive	Chronic infection well spread in the udder	A3
1	2	Chronic infections/subclinical mastitis	Intermittent shedding with varying frequency	Could be low grade infection spreading through the udder or with genotype specific virulence factors	A4
1	3	Healthy	No infection, low SCC		A1
1	4	Transient infection (probably)	Short-lasting peak	Quick infection. Reaction to infectious agent and then most likely killed	A8
2	1	Subclinical mastitis	The curves are steady	The sample reflect a permanent infection	A3
2	2	Healthy animal	The curves show low/no amount of bacteria	Healthy animals with no further problem with bacterial infections. Maybe sample issue in quarter 11 and 12	A2
2	3		Blue line goes up when green line goes down		A10
2	4	Clinical mastitis	High culture counts, relatively low Ct-values		A3
3	1	Negative quarters	Negative quarters to *Staph. aureus*	No *Staph. aureus* mastitis	A1
3	2	Positive - intermittent	Positive quarters with intermittent elimination of the bacteria	Infected quarters, showing a shedding pattern. Intermittent. Therefore, elimination in milk and diagnose is intermittent. Also the SCC - could be related with chronic infection.	A4
3	3	Positives - steady	These quarters were positive and remained positive across the study	Infected quarters, showing a steady elimination of the bacteria and steady high SCC (>100,000). Acute infection.	A3
4	1	*Staph. aureus* negative	PCR negative, BC negative - except day 3, quarter 12. SCC low - except quarter 36	Unaffected quarter – *Staph. aureus*	A2
4	2	Quarter infected with *Staph. aureus*	PCR positive, BC positive, SCC medium to high	Infected quarter	A3
4	3	Intermittent quarter, *Staph. aureus*	PCR mostly positive, BC variable/positive, SCC medium/high	Intermittent shedding of *Staph. aureus*	A4

4	4	Recurring contamination with *Staph. aureus*	PCR mostly negative, BC variable, SCC medium	Intermittent shedding or environmental/other reservoir on skin/teat end	A4
6	1	Cured infection	Positive PCR and culture on one moment in combination with a high cell count	The cow was infected and got cured very easily	A9
6	2	More chronic infection	Constantly positive PCR and culture in combination with high cell count	The immune system has trouble dealing with the *Staph. aureus* and can't get the infection under control. *Staph. aureus* is probably not moving intracellular	A3
6	3	PCR gives some negative results while culture is positive	Slightly elevated cell count, culture is constantly positive but PCR sometimes give a negative result		A10
6	4	Recurrent infection	Elevated cell count with alternating positive and negative PCR and culture results	*Staph. aureus* is hiding intracellular until the immune system is getting less active	A4
8	1	Chronic	Treat at dry off or cull	The cow cannot control the infection	A3
8	2	Healthy	Do nothing	The cow seems to control the infection	A1
8	3	Chronic-dynamic	Infection status varies over time. Do nothing	The immune system is trying to control the infection	A4
9	1	Chronic infection, high shedder	More or less consistently high cfu count + low Ct-value	If I had an additional envelope, I would have split this into highly virulent (elevated SCC) and low virulent (low SCC). I considered a separate envelope for teat canal colonization, but the presence of *Staph. aureus* is more persistent than I have previously seen for teat canal colonization so I'm calling them all infections	A3
9	2	Chronic infection, variable shedding	Persistent infection with variable bacterial shedding levels	Bacteria trapped in micro abscesses or blocked ducts, persistently present in mammary gland but not always present in milk	A4

9	3	Transient infection	Temporary presence of *Staph. aureus* and SCC elevation	Infection (based on positive PCR and/or culture) followed by influx of neutrophils (strong increase in SCC) with successful removal/killing of bacteria. quarter 12 early in this process -> bacteria still viable. Quarter 36 late in this process -> bacteria already killed	A8
9	4	*Staph. aureus* free	Negative or positive PCR result but no viable bacteria and no increase in SCC.	No infection or colonization with *Staph. aureus*, quarter 35 false positive PCR e.g. due to skin flora	A2
10	1	Healthy cow	Short-lasting shedding	Short and heavy shedding	A8
10	2	Infected cow	Varying shedding where PCR/BC is negative at the same time	Short-lasting missing shedding	A4
10	3	Chronic infected	High shedding – possible to identify on BC/PCR daily	Varying shedding, but with continuous shedding	A3
10	4	Healthy cow	Low shedding, Ct-value agrees with neg BC	Cow with short reaction in PCR -> with same reaction in SCC	A8
12	1	Non-infected	No growth, high Ct (occasional positive in some samples - wrong?)	Typical. One single + does not matter - maybe inf. Comes and goes. I wouldn't mind.	A2
12	2	Persistent infection, high shedding	High cfu all the time. Not always high SCC. Ct occasionally high	not exceptional model for *Staph. aureus*	A3
12	3	Persistent inf. Low shedding (low CFU occasionally)	High cfu mainly, but drops. SCC mainly high	High CFU mainly, but drops. SCC mainly high	A3
12	4	Transient infection	Cfu rises and drops, so does SCC	Possible in *Staph. aureus* infection. Ct 37-40 is not any more reliable (Ct cut-off 37 only preferable)	A8
13	1	Healthy, non-SA quar	Culture and PCR negative, low SCC	Non-infected, low SCC quarter, some variation in SCC (maybe heat-induced?)	A1
13	2	High shedder	Consistently high CFUs and low PCR -> plenty of ? To be trend?	Infected *Staph. aureus*-quarter with elevated SCC	A3
13	3	Intermittent shedder	Both + and - culture and PCR results with daily variation also in SCC		A4

13	4	Steady shedder (slight variation)	(Mostly) culture positive (a few quarters with one day negative) also mostly PCR positives, however, more variability in PCR (more >PCR - than culture - for which I do not have an explanation) SCC elevated	Infected *Staph. aureus*-quarter with somewhat elevated SCC	A5
7	1	*Staph. aureus* infected	PCR+, SCC+, culture +		A3
7	2	Infected, intermittent shedding	PCR+/-, SCC +/-, culture +/-		A4
7	3	Non-infected			A1
7	4	Intermittent shedding			A4
14	1	No problem/healthy	PCR negative, culture negative, no high SCC		A1
14	2	Acute infection	Culture +, PCR highly +, SCC: no significant changes	Active infection	A3
14	3	Inconclusive	PCR +/-, culture +, SCC: no alterations	Intermittent excretion, contaminated samples	A10
14	4	Active infection	PCR + almost all time, culture +, SCC: no biological changes		A5
15	1	Contaminated sample	Healthy quarters - sampling contamination possible in some cases where transient increases in SCC/PCR + culture	Low levels of infection or sample contamination	A2
15	2	Chronic infection	Chronic stable infection	Culture + / stable SCC/ PCR +, stable for 21 days suggest a chronic but stable infection	A3
15	3	Uncontrolled infection	Uncontrolled infection	fluctuating PCR + increasing culture	A5
15	4	Low infection levels	Stable SCC/fluctuating culture/PCR?	Low level infection	A4
16	1	Healthy	BC neg, most of them low SCC	Other source of *Staph. aureus*?	A2
16	2	Persistent subclinical Infection	High SCC, BC+, PCR +	Persistent, invasion of teat/quarter, immune system not able to get rid of BC	A3
16	3	Subclinical cows. Intermittent shedding	Shedding/immune response, pattern	Shedding/immune response, pattern	A4
16	4	Subclinical	Constantly shedding until day 9-10, then a "dip", then shedding	Immune response gets rid of BC at day 9-10 then comes back	A4

17	1	Chronic carrier	Healthy animal	Presence detected, but staph is hidden in the parenchyma is not shedding it	A10
17	2	Subclinical mastitis chronic carrier	Chronic carrier and contagious pattern	There is impairment in the udder, troubled by increased SCC, staph. is detected is shedding it	A3
17	3	Chronic clinical mastitis	Contagious pattern		A3
17	4	Chronic recurrent infection		The animal dealt with the infection and have a recurrent infection	A4
18	1	No treatment or preventive medicine	No excretion of *Staph. aureus*, SCC mainly below 200,000	Environmental infection, reff control	A1
18	2	Treatment: culling of chronic cows, separation of other infected	Persistent infection with high SCC values	Contagious infection, *Staph. aureus* persist in the udder, chronic inflammation	A3
18	3	Dry cow therapy for cows with low SCC, culling for the other	Intermittent shedding both in PCR and culture, somatic cells mainly > 200,000	Contagious infection with intermittent shedding	A4
18	4	Unknown	Unusual pattern of excretion, different response between PCR and culture		A10
19	1		Slightly variable in culture result, positive throughout period, variability in PCR result. SCC mostly consistent, slight variability in data. Culture + over 4 (log 10) cfu/ml, PCR +, SCC generally over 2 (log scale)		A3
19	2	PCR - and culture -	PCR negative and culture negative (in general). 1 case where this doesnt fit patterns I grouped (quarter 37)		A2
19	3	Irregular data pattern (very variable data)	Culture results highly variable, SCC greatly variable, PCR very variable, SCC and PCR highly conflicting results		A10
19	4	Over 4 CFU/ml culture results, variable PCR results with "spikes" in data	Data patterns that didn't fit my other choices. Mainly related due to peaks in PCR ct value, e.g.: negative periods. Mostly positive PCR results but variable		A10

			over time. SCC 2 or above, culture + over 4 cfu/ml		
20	1	Healthy	Low (normal) SCC, negative PCR, negative culture	Quarter 12 spontaneous cure or mixing of sample results?	A2
20	2	Intermittent shedding accord. Culture	Heavy shedding and no growth on agar, PCR+ or PCR -, SCC high	Intermittent shedding but why PCR negative? Check the PCR curves at least for quarter 11	A4
20	3	Constant shedding I	BC+/PCR+ with minor number of PCR neg samples	Growth ability of *Staph. aureus*reus ? Good, constant shedding. Why the PCR - ?	A5
20	4	Constant shedding II	PCR+, BC+	Truly infected cows according every measurable meters?	A3
21	1	Low shedders	Low SCC, almost no shedding of *Staph. aureus*, maybe one flare-up	Transient infection	A8
21	2	Varying shedding - low SCC	Shedding varies a lot, sometimes being very high. SCC is usually low	Infection	A4
21	3	Varying shedding - high SCC	Shedding varies a lot, between low and very high from day to day, SCC is high	This might already be clinical mastitis (we do not know from these tests), severe infection	A4
21	4	Consistent mild shedders	SCC 200-400,000, consistent shedding pattern, mild infection	Chronic infection	A3
23	1	Chronic infection intermittent shedding	The SCC is elevated throughout the study period. *Staph. aureus* is shed intermittently. Cannot explain one where *Staph. aureus* is not detected by PCR and by culture. Perhaps the quarter is almost succeed to get rid of the pathogen?	Chronic infection of the quarter(udder) (with intermittent shedding)	A4
23	2	Acute episode of mastitis	Acute episode of *Staph. aureus* mastitis with SCC affected	Acute infection which is rapidly resolved	A8
23	3	Quarter diagnosis of *Staph. aureus* was not confirmed	No *Staph. aureus* detected for the study period. This is a healthy udder.	No mastitis	A1

23	4	Chronic infection	The udder/quarter is infected throughout the study period - more or less constant shedding. Elevated SCC. Culture + PCR corresponds	Chronic infection, continuous shedding of bacteria	A3
24	1	Healthy cows	Low cfu, low SCC, high ct	Healthy cows without infection	A1
24	2	Intermittently shedding	Varying cfu, high ct, medium SCC	Strain that is intermittently shed in the milk (or flushed) but without trace in PCR and SCC	A4
24	3	Permanently infected	High cfu, high SCC, low ct	Cows that are permanently infected and the pathogens are seen in the milk	A3
25	1	Healthy	Low SCC, Ct 40, no growth (mostly) - I would have liked a group for this	Healthy	A2
25	2	Positive	SCC at cut-off or higher, low Ct, a lot of growth	Infected	A3
25	3	Unclear diagnosis	High SCC, mostly PCR positive, really unstable growth	No idea	A10
25	4	Unclear culture, PCR negative	SCC around cut-off, PCR negative, unstable growth	No idea	A10
26	1	Infected	These are infected cows	The reasons are: SCC>100,000 most of the time, BC is high most of the time, PCR-ct is low most of the time	A3
26	2	Negative cows	These are cows that do not have mastitis (IMI)	SCC<100,000, bc negative, ct values constantly high	A1
26	3	Latent cows	These are cows that have the pathogen but the pathogens have not yet established a clear infection. The cow is hiding the infection	SCC varies around 100,000 cells/ml, bc changes frequently, ctvalues change frequently	A4
26	4	Non-infection reaction	A reaction in the SCC that is not a result of an IMI causing pathogen	Only increase in SCC > 100,000 cells/ml, BC negative, only slight reduction in ct one time	A2
28	1	Non infected cows	BC -, PCR -, SCC low	Cows not infected with *Staph. aureus*. Low SCC therefore healthy mammary gland	A1

28	2	Persistently infected cows	BC + for all the study, PCR +, SCC high, low or fluctuating. If PCR fluctuation or negative and PCR + considered infection	Likely to be cows infected with a contagious *Staph. aureus* strain. Confirm with strain typing this. Advice measures to control contagious mastitis	A5
28	3				A10
28	4	Fluctuating infection. BC and PCR matching, positive at some time points			A4
29	1	Ongoing "chronic" infection	Infection + inflammation over 3 week period	Evidence of viable bacteria supported by positive PCR over 3 weeks, with consistent elevation of SCC	A3
29	2	Uninfected	No evidence of infection or inflammation	consistent failure to demonstrate presence of bacteria (viable (based on culture) or DNA (based on PCR)) SCC consistent below threshold of positive	A1
29	3	Acute infection	Only infected for very brief period	Apparently "normal" except for a discreet 24 hour period (single sample) in which bacteria was present. Accompanied by increase in SCC	A8
29	4	Chronic active infection	Inconsistent shedding of bacteria but evidence of ongoing inflammation	Bacteria only intermittently shedding in milk. Some inconsistency between culture and PCR due to one testing viable bacteria + one detecting DNA (viable or non viable bacteria). SCC indicative of ongoing inflammation	A4
30	1	Healthy	Low SCC, no or only transient IMI	Cows manage to clear infection or don't get infected.	A8
30	2	*Staph. aureus* IMI	Intermittent shedding, maybe hard to diagnose	*Staph. aureus* may remain intracellular	A4
30	3	*Staph. aureus* IMI, high shedders and responders	easy to detect by SCC/PCR/culture	Constant shedding, constant PMN immigration	A3
30	4	Questionable *Staph. aureus* IMI	SCC may point at *Staph. aureus* IMI	May be very well adapted strain? On PMN (SCC) phagocytosis all the	A10

				bugs	
31	1			More or less healthy udder quarters	A2
31	2			*Staph. aureus* induced mastitis, *Staph. aureus* more or less constantly released	A4
31	3			intermittent shedding of *Staph. aureus*, correlating with intermittent raised SCC	A4
31	4			healthy udder quarters despite the presence and shedding of *Staph. aureus*	A2
33	1	Uninfected	No micro culture, low SCC, negative PCR	Uninfected normal quarter	A1
33	2	Infected with another pathogen/transient infection	Negative/mostly negative PCR. Negative/contaminated (?) culture. Blips of high SCC	Transient infection w. staph aureus. -/+ infected w. another pathogen	A8
33	3	Infected with mostly low SCC	Micro culture +. Mostly PCR +, low (mainly) SCC	Low immune responders?	A5
33	4	Infected with mostly high SCC	Micro culture + (or intermittent) Mostly PCR +, mostly high /blips of SCC	Possible intermittent shedding or highly infected quarter	A4
34	1	Inconclusive	SCC results, PCR values + culture results in conflict - unable to conclusively diagnose infection when all 3 considered	Possible reasons: contamination of plates? Validity/accuracy of PCR testing? Infection with other organisms?	A10
34	2	Chronic infection with persistent shedding	SCCs continually raised, repeat positive culture results for *Staph. aureus* + repeat positive PCR	Continual presence of *Staph. aureus* triggering immune system + production of WBC	A3
34	3	Chronic infection, intermittent shedding	Persistently high SCC, repeat positive culture + PCR results but not consistent	Intermittent shedding maybe a reflection of bacterial (type?) behavior, or cows immune system/ability to clear detectable bacteria? However, would the latter be sufficient/effective enough to remove all traces of DNA?	A4
34	4	Resolved or uninfected	Persistently normal/low SCC, repeat negative culture. Apart from transient infection		A8

			period		
35	1	Healthy quarter	No shedding of *Staph. aureus*, low SCC	Self-cure/treatment successful	A1
35	2	Established infection, continuous shedding	constant shedding, PCR positive, culture positive, high SCC	infection established -> balance between bacterial growth and immune response -> lag-phase	A3
35	3	Intermittent shedding, established infection	Positive and negative PCR/culture intermittent, SCC high	*Staph. aureus* might be encapsulated and therefore shedding is intermittent	A4
35	4	Non conclusive shedding	Positive culture over whole time period, intermittent PCR positive	No idea	A10

Streptococcus agalactiae					
Expert ID	**Envelope #**	**Label (Expert)**	**Description (Expert)**	**Biology (Expert)**	**Code (authors)**
1	1	Healthy + one transient infection	No infection or transient		A10
1	2		PCR positive only but low Ct-values off and on AND SCC -> likely infection/subclinical mastitis		A5
1	3	Infection. Subclinical mastitis	Persistent infection with increased SCC		A3
1	4	New infection -> chronic infection and subclinical mastitis			A6
2	1	Healthy animals	Ct-values are high, cultures are low, SCC steady	No infections that cause problems	A1
2	2	Clinical mastitis	Low Ct-value, high culture no., high inflammation SCC	Infection	A3
2	3				A10
2	4	Clinical mastitis	Curves are going up/down	Infection and treatment	A4
3	1	Negative quarters	Negative quarters to *Strep. agalactiae*	No mastitis caused by *Strep. agalactiae*	A1
3	2	Positive - steady	Positive quarters. Across the study	Infected quarters with continuous shedding of the pathogen and steady high SCC. Contagious pattern.	A3
4	1	*Strep. agalactiae* negative quarters	PCR negative, BC negative, SCC<100,000	Normal udder	A1
4	2	Quarters negative on BC	PCR variable, BC negative, SCC low/high	Environmental strains?	A2
4	3	Quarters infected by *Strep. agalactiae*	PCR positive (mostly), BC positive (mostly), SCC high	Persistently infected quarters	A5
4	4	Newly infected with *Strep. agalactiae*	PCR (mostly) positive - after 7 days, BC (mostly) positive after 4 days, SCC increasing	Cow infected early in 21-day period	A7
6	1	Likely negative quarters	Culture is negative/SCC is low, PCR is negative, has one small peak	The infection cured very easily (non-udder adaptive strains?)	A8
6	2	Culture negative quarters	Culture is negative but cell count is high and the PCR is alternating positive and negative		A10
6	3	PCR or culture negative on one or several point	SCC is (mostly) high, the PCR and culture mostly positive	This strain has probably the ability to fool the immune system	A5
6	4	Constantly positive	High SCC, positive	Contagious strain	A3

		PCR and culture	culture and PCR		
8	1	Chronic	Cull		A3
8	2	Healthy	Do nothing		A1
8	3	Infected, flare ups	Treat if high yielding, otherwise cull		A4
9	1	Chronic infection	Persistently positive for *Strep. agalactiae* as evidenced by culture and/or PCR	Persistent intramammary infection. Quarter no. 21 new IMI, established by day 5, quarter no. 13 one false negative culture result - possibly sampling, lab or recording error	A5
9	2	Healthy quarter	No evidence of *Strep. agalactiae* infection		A1
9	3	Clinical irrelevant	Single PCR positive with very limited and short-lived SCC elevation (barely above 100,000 cells/mL)	False positive PCR/mislabeling, transient infection, very short-lived, fecal contamination, human *Strep. agalactiae* contamination	A2
9	4	A riddle	Culture negative, repeatedly PCR positive (>=3). SCC elevation >100,000 cells/mL	Low level infection with neutrophils keeping it in check by killing *Strep. agalactiae* (hence PCR+, culture -). *Strep. agalactiae* with specific growth requirements, not met by standard growth conditions in the lab or not recognized, e.g. CAMP negative (false negative culture results)	A5
10	1	BC negative	SCC > 100,000 and varying PCR positive	Earlier chronic infection with shedding of dead bacteria	A9
10	2	Low SCC	Cow with minimal shedding	Immune system defends infection?	A10
10	3	Varying shedding	PCR/BC do not agree	Intermittent shedding -> hard to identify with 1. sample	A4
10	4	Chronic	Continuous shedding with positive BC/PCR	Cows with increased SCC and continuous positive BC	A3
12	1	Non-infected	Low Ct-value, no growth, mainly low SCC, occasional low Ct-value - contamination, carry over?	Nothing special, free of infection	A2

12	2	Infected permanently	Mainly high CFU and low Ct-values and high SCC	Typical *Strep. agalactiae* infection, heavy infection. Occasional no-growth sample does not matter	A5
12	3	Possible infection low cfu	CFU detection limit too low, PCR detects possible infection, SCC high -> supports presence of IMI	Not so common, possible	A5
12	4	New infection	First negative, then positive	This happens	A6
13	1		Culture negative quarter, mostly also PCR negative	Positive *Strep. agalactiae* PCR results, but nothing in culture maybe explained by the cow eliminating the bacteria spontaneously	A10
13	2	*Strep. agalactiae* positive, high consistent shedding	high cfus, low Ct-values, elevated SCC	Infected quarter	A3
13	3		Mostly positive cultures and PCR results, elevated SCC	*Strep. agalactiae* infected quarter. Some (2) newly infected quarters	A10
7	1	*Strep. agalactiae* infected	high SCC, PCR+, culture +	Chronic	A3
7	2	*Strep. agalactiae* infected	PCR +, SCC+, culture + almost every time		A5
7	3	Non-infected quarter	PCR-, SCC-, culture -		A1
7	4	Non-infected quarter	PCR sometimes +, SCC+, culture -		A2
14	1	Contaminated milk?	PCR-, culture+, SCC: stable	Active infection	A10
	2	Infected	PCR+ with negative peaks, culture: positive, SCC: high	Presence of bacteria	A5
14	3	Healthy	PCR-, culture -, SCC: low	No problem	A1
15	1	Low level infection after day 11	Culture -, SCC -, PCR towards negative	Low level infection after day 11	A10
15	2	Stable chronic infection	Culture positive stable, stable low SCC, PCR +	Stable chronic infection	A3
15	3	Acute infection	PCR +, culture +, SCC+	Active infection	A3
15	4	Low controlled infection	Low level of infection not picked up by culture	Low level of controlled infection	A4
16	1	Persistent subclinical	SCC > 100,000, BC+, PCR + throughout period	Subclinical cows	A3
16	2	Healthy	Low SCC, PCR negative most of the time, BC negative throughout the period	Initial response to *Strep. agalactiae* challenge	A2
16	3	Subclinical mild	BC negative, SCC>100,000, PCR+,	High SCC for other reason than *Strep.*	A10

			throughout period	*agalactiae*? Carry over?	
16	4	Clinical	SCC initially low -> increase ->PCR+ Ct-value decrease, CFU BC increase	Initial challenge, immune response tries to get rid of bacteria and almost succeed. Bacteria "wins"	A6
17	1	Carrier of *Strep. agalactiae*		The animal has *Strep. agalactiae* - is a carrier but there is no inflammation and no shedding of bacteria	A5
17	2				A10
17	3				A10
17	4				A10
18	1	No treatment, self-recovery of the animals	Low SCC, no excretion	Self-cured cow	A1
18	2	Culling of chronic cows and treatment of cows lower SCC	High excretion of *Strep. agalactiae*, high SCC	Contagious chronic infection	A3
18	3	Culling of chronic cows and treatment of cows lower SCC	SCC higher than 100,000, intermittent excretion of *Strep. agalactiae*	Contagious infection	A4
18	4	Treatment probably non effective	No detection in culture, only detection by PCR, SCC high	Low excretion but presence of inflammation, environmental infection possible	A10
19	1	PCR +, high SCC	SCC over 3, SCC and culture pattern cross one another (overlapping data lines). PCR generally below 25 (reference line) positive		A10
19	2	Culture negative	Culture negative, PCR negative		A10
19	3		PCR dramatically changes/greatly increased at specific time points - over 35 ("erratic") PCR results. SCC generally over 3 (log scale), quite erratic results		A10
19	4	Culture +, PCR -, SCC generally under 2	3 distinct data lines - no overlapping of SCC/culture/PCR. PCR >25 almost always. SCC over 2, relatively stable culture results (on log scale). Culture +, PCR -.		A10
20	1	Healthy quarters	Low SCC, negative PCR, culture -	No infection	A1

20	2	BC-, PCR +	Culture negative, PCR +, high/variable SCC	Indication of inf. Present according the SCC. Intermittent shedding or contaminations?	A4
20	3	PCR+, BC-	Nice agreement on PCR and BC results	Constant shedding of *Strep. agalactiae*, probability of "truly infected" is high even based on just PCR results. Others more consistent than others, but still constantly heavily infected cows.	A3
20	4	The annoying ones	Indication of pathogen present according SCC, but rather powerful disagreement on some sampling points. Even could suspect of errors in some samplings: the sampling, handling, should check the PCR curves; was there some bad ones?	Can't explain everything…	A10
21	1	Persistent infection	High SCC, high and consistent shedding	Persistent infection	A3
21	2	Infection by PCR	High SCC, culture -, PCR often positive	Infection, the question is why is culture not?	A5
21	3	Healthy	Low SCC, no shedding	Healthy, one transient finding, maybe contaminant	A2
21	4	Starting infection	SCC starts to rise and shedding increases suddenly	Starting infection	A6
23	1	Chronic infection	Chronic most probably subclinical infection - elevated SCC throughout. More or less continuous shedding - these cows are contributing to spread! (cull)	Chronic infection with bacterial shedding	A5
23	2	Healthy udder quarter	No infection, no shedding	Healthy quarter	A1
23	3	Not sure	Much higher Ct-values than the chronic infections - occasional detection by PCR but no culture, yet cell counts are elevated. Could be false positive PCR (environmental, contamination or carry over) or low grade infection with some	Not sure	A10

			effect on SCC and intermittent shedding		
23	4	Acute resolved	PCR positive with elevated SCC at day 3 - then resolves. Never culture positive	Acute infection resolved	A8
24	1	Persistently infected	High CFU, high SCC, low ct	The quarter is infected persistently, throughout the study. Some errors with the measurements occur	A5
24	2	Not infected	Low CFU, low SCC, high Ct-value	No infection in the quarter	A1
24	3	Starting to be infected	High PCR, low CFU, high SCC	Newly infected quarter where SCC has gone up due to the infection but bacteria are not shed in the milk yet	A6
25	1	Positive - steady	High SCC, low ct, lots of bacteria	Infected	A3
25	2	There is nothing/healthy	Low SCC, Ct-value 40, no growth	No *Strep. agalactiae*/healthy	A1
25	3	Only PCR positive	high SCC, sometimes positive PCR, no growth	Was infected?	A9
25	4	Status change	Goes from unclear (mostly low SCC, high Ct-value, little growth) to high SCC, low Ct-value, lots of bacteria	Change in infection status	A6
26	1	Infected	These are infected cows (IMI)	SCC>100,000 most of the time, BC is high most of the time, Ct-values are low most of the time	A5
26	2	Negative cows	Cows free from IMI	SCC<100,000 constantly, BC very low, Ct-value high most of the time	A2
26	3	Latent cows	These are cows that have the pathogen but the pathogen had not established clear infections. The cows immune system is fighting hard	SCC varies around 100,000, BC changes frequently, Ct-values changes frequently	A4
26	4	Non-infection reaction	A reaction in the SCC that is not a result of an IMI causing pathogen	Only increase in SCC >100,000, BC negative mostly, PCR Ct-values have only a limited and single reaction	A2

28	2	Persistently infected cows. Culture + during all the study, cows persistently shed bacteria	BC +, PCR +, SCC high or fluctuating	Likely to be cows infected with contagious *Strep. agalactiae* strain. Confirm this with strain typing. Advise the farmer to put in place measures to control contagious mastitis	A3
28	3	Culture negative cows with PCR + samples at some time points, with peaks or increase of SCC. Cows intermittently shed bacteria	Cows are culture negative throughout the period but have PCR + at some time points and occasional increase in SCC	These cows may be infected and/or shed with low bacteria numbers thus the negative count BC increase. The PCR may pick these. Advise the farmer to investigate the strain involved to see if it is a contagious (same strain over time) and in this, put in place measures to control contagious mastitis or if environmental in this case advise measures for environmental mastitis	A4
28	4	Non infected low SCC (- BC, -PCR)	Cows non infected with *Strep. agalactiae*. Low SCC therefore they probably do not have other infections, they do not require treatment. Advise the farmer to keep going with good management and milking practices.		A1
29	1	Not infected	Consistent absence of live/variable bacteria	No living bacteria present. PCR indicates no or only transient existence of *Strep. agalactiae* DNA	A2
29	2	Newly infected with *Strep. agalactiae*	Initially lacking *Strep. agalactiae* bacteria but bacteria subsequently established	Bacteria introduced early to an apparently uninfected gland. Subsequently established infection with evidence of live, viable bacteria	A6
29	3	Misdiagnosis	Bacteria consistently present except for a single unexpected "no growth"	Likely to be an ongoing infection based on PCR, cell count and culture. A single day of no growth likely to be due to laboratory failure as PCR remains +	A5

29	4	Ongoing infection	Viable bacteria consistently growth in culture	Established infection with repeated positive culture and positive PCR and associated evidence of inflammation based on SCC (elevated)	A3
30	1	*Strep. agalactiae* IMI	High SCC and shedding. Easy to diagnose	Constant shedding, constant PMN immigration	A3
30	2	*Strep. agalactiae* IMI	Intermittent shedder	High SCC may lead to intermittent shedding by phagocytosis (PMN)	A4
30	3	Hard to detect	Culture negative, high SCC, PCR intermittent	IMI but very few bacteria	A5
30	4	No IMI	Healthy cow, may have false positive PCR	Healthy cow, may have false positive PCR	A2
31	1			Healthy udder quarters, *Strep. agalactiae* eliminated	A1
31	2		Constant presence of *Strep. agalactiae* together with constantly raised cell number	*Strep. agalactiae* causative for the mastitis, constantly triggers influx of somatic cells	A3
31	3			Periods of mastitis, *Strep. agalactiae* initially present but not the causative agent	A10
31	4			Infection with *Strep. agalactiae* - day 3/day 4 -> mastitis up to day 19	A8
33	1	Infected	Micro culture +, PCR overwhelmingly +, SCC moderate to high	Chronically infected quarters	A3
33	2	Uninfected/transiently infected	Micro culture -, PCR negative (but one blip), low SCC	Uninfected, perhaps one blip of transient infection	A8
33	3	Infected with another pathogen	Culture -, PCR mostly -, SCC moderate to high.	Free from *Strep. agalactiae*, infected with another pathogen	A2
33	4	Clearing/low level infection	Micro culture -, PCR begins +, then goes -. SCC starts high, then decreasing	Early infection then clears (missed with culture)	A9
34	1	Resolved/uninfected	Repeatedly normal SCC + negative culture/PCR		A1
34	2	Chronic infection, persistent shedding	Between SCC/ culture/ PCR, evidence of contaminated (or unresolved) infected status, with continual evidence of bacteria present		A3

34	3	Inconclusive	Evidence of infection as determined by SCC, culture and/or PCR but conflicting diagnosis depending on tool used	Possible reasons: contamination of plates? Validity/accuracy of PCR testing? Infection with other organisms?	A10
35	1	Healthy quarter	no shedding of Streptococcus over 21 days	Quarter spontaneously cured or after treatment	A1
35	2	Shedding non vital Streptococcus -> post treatment/during treatment	Only pos PCR intermittently, neg culture	Quarters treated or cured, shedding of non-vital bacteria, positive in PCR/ negative in culture	A9
35	3	Establishing an infection	Increase of shedding over 7 days, elevated level afterwards	Lag-phase of infection	A6
35	4	Established infection	Constant shedding of Streptococcus	Balance between bacterial growth and immune system lag-phase of infection	A3