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Multiplex Real-Time PCR in Bovine Mastitis Diagnostics

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ACADEMIC DISSERTATION

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Mummolle.

Dedicated to my beloved late grandmother who waited for this day more than anybody else.

Abstract

Mastitis is the most common and economically damaging production disease of dairy cows. In the majority of cases it is induced by intramammary infection with a pathogen. Identification of mastitis-causing pathogens enables treatments to be targeted appropriately, avoiding unnecessary antimicrobial treatments, and enabling design of the best possible herd level management protocols. Diagnosis of bovine IMI has been traditionally based on microbial culture of aseptically taken quarter milk samples. Due to the requirements of modern dairy production and development of molecular technologies, milk samples can be examined for target pathogens with DNA-based methods such as multiplex real-time PCR assays. Despite several years of routine use of PCR assays in some countries, scientific knowledge concerning interpretation of the results is sparse.

This dissertation focuses on providing new information for diagnosing bovine mastitis using multiplex real-time PCR. In order to accomplish that, first a commercial multiplex real-time PCR assay was compared with the current reference method, conventional culture (I). It showed that PCR was a reliable diagnostic method for *Staphylococcus aureus*, but less reliable for diagnosing true non-aureus staphylococci infections. In an experimental challenge study (IV), elimination of *Staphylococcus epidermidis* and *S. simulans* was followed with PCR in addition to conventional culture. The results showed that PCR yielded target-positive results for several days after the culture results were negative. Subsequently, the time of sampling related to the onset of inflammation may be of importance when interpreting the PCR results.

Secondly, quarters with suspected mastitis were sampled using two experimental techniques, in addition to the conventional aseptic technique (II, III), and examined with multiplex real-time PCR. In the needle technique, the whole teat and teat area were bypassed. With the cannula technique, the teat orifice and the teat canal were bypassed. The results showed that samples taken with alternative techniques were of better hygienic quality. We also reported that over half of the non-aureus staphylococci findings in our PCR results originated from sites other than the mammary gland.

A diagnosis of a true IMI or mastitis, with possible outcome of an antimicrobial treatment course for the animal, cannot be based solely on a PCR result, but must be combined with all the available information about a cow's history, symptoms, changes in milk and indicators of inflammation. Some microbes detected with PCR can be considered to be contaminants, especially when present with low levels of DNA. If the signs of the cow or the degree of udder inflammation do not fit the usual signs caused by the pathogen detected, the possibility of contamination should be considered.

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I will remember the first call as a veterinarian for the rest of my days. The caller was a man whose cow had mastitis. He had taken an aseptic quarter milk sample and the result was *Str. dysgalactiae* with three plusses. I knew the pathogen, I knew the treatment but what did the three plusses mean? I would have never started this PhD without the genuine need for information on how to interpret the PCR results. Thus, I want to thank each and every client of mine during these years – you were the motivation behind all this research.

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List of original publications

This thesis is based on the following publications:

- I Hiitiö H., Riva R., Autio T., Pohjanvirta T., Holopainen J., Pyörälä S. and Pelkonen S. 2015. Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture. *J Dairy Res.* 82:200-208.
- II Hiitiö H., Simojoki S., Kalmus P., Holopainen J. and Pyörälä S. 2016. Effects of sampling technique on the PCR-based microbiological results of bovine milk samples. *J Dairy Sci.* 99:1-10.
- III Friman M., Hiitiö H., Niemi M., Holopainen J., Pyörälä S. and Simojoki H. 2017. The effect of a cannula milk sampling technique on the microbiological diagnosis of bovine mastitis. *Vet J.* 226: 57-61.
- IV Hiitiö H., Pyörälä S., Taponen S., Rajala-Schultz P. and Simojoki S. Elimination of experimentally induced bovine intramammary infection assessed by multiplex real-time PCR and bacterial culture. *J Dairy Sci.* 101:5267-5276.

The publications are referred to in the text by their Roman numerals. The original articles are reprinted with the permission of the copyright holders: Elsevier B. V. (the *Journal of Dairy Science* and the *Veterinary Journal*) and Cambridge University Press (the *Journal of Dairy Science*).

Abbreviations

AMS	automatic milking system
BC	bacterial culture
<i>blaZ</i>	beta-lactamase gene
BTM	bulk tank milk
cfu	colony forming unit
CM	clinical mastitis
CNS	coagulase negative staphylococci
Ct	cycle threshold
gc	genome copy
DNA	deoxyribonucleic acid
dNTP	deoxy ribonucleotide triphosphate
EC	electrical conductivity
IAC	internal amplification control
IMI	intramammary infection
LCA	latent class analysis
NAGase	N-acetyl- β -D-glucosaminidase
NAS	non-aureus staphylococci
<i>nuc</i>	gene, which encodes the thermostable nuclease of <i>Staphylococcus aureus</i>
PC	post challenge
PCR	polymerase chain reaction
pH	potential of hydrogen
pmol	picomole
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCC	somatic cell count
SCM	subclinical mastitis
Se	sensitivity
Sp	specificity
WHO	World Health Organization

1 Introduction

Mastitis is defined as an inflammation of the parenchyma of the mammary gland (Zhao and Lacasse, 2008), a local protective reaction against irritation (International Dairy Federation, 2011) of any possible cause. The most common cause of mastitis is intramammary infection (IMI), defined as the presence of pathogenic micro-organisms in the secretory tissue and the duct and tubules of the bovine mammary gland (International Dairy Federation, 2011).

Mastitis decreases the ability of the mammary gland to produce milk. Concentrations of milk components, such as fat and protein, decrease and proteolytic activity increases, leading to poor milk quality (Seegers et al., 2003). Mastitis causes pain and discomfort to affected cows (Fogsgaard et al., 2012; Sepúlveda-Varas et al., 2014; Fogsgaard et al., 2015). In addition, mastitis is one of the major reasons for antimicrobial use in dairy cattle (Erskine et al., 2003; Halasa et al., 2007; Barlow, 2011). Prevalence of bovine mastitis varies across countries between 19 – 53% (Pitkälä et al., 2004; Østerås et al., 2006; Haltia et al., 2006; Hiitiö et al., 2017) and is still the most common disease among dairy cows. Production losses, discarded milk, treatment costs, increased labour, premature culling and replacement costs (Halasa et al., 2007; Heikkilä et al., 2012) also make mastitis the most economically damaging production disease of dairy cows (Seegers et al., 2003).

Mastitis-causing pathogens may originate from the environment, milking equipment, barn environment, udder, or skin of the cow or humans. Udder pathogens may in some herds be of a more contagious nature, and in some herds environmental, requiring different approaches for herd mastitis control. IMI is the consequence of a failure of the first line defence mechanisms, i.e. mechanical barriers of the teat. If the subsequent cellular and humoral defence mechanisms also fail, mastitis is induced (Zhao and Lacasse, 2008). Mastitis can be clinical or subclinical. In clinical mastitis, pathological changes to the glandular tissue cause the typical inflammatory signs: swelling, heat, pain, redness and loss of function (Radostits et al., 2007). The alveoli and the mammary gland lose their structure and the blood-milk barrier is disrupted (Zhao and Lacasse, 2008), leading to visible changes in the milk, such as discoloration and clots or flakes. Mastitis can sometimes even be fatal. During IMI and mastitis, the number of polymorphonuclear leucocytes, comprising the majority of the somatic cells in the milk, increases dramatically. The primary change in subclinical mastitis is elevated milk somatic cell count (SCC), which can be observed with several cow-side and automatic detection methods.

Different microbes harbour different virulence factors and induce mastitis with different degrees of severity of tissue damage and different prognoses (Zhao and Lacasse, 2008; Schukken et al., 2011). Susceptibility to antimicrobials and treatment response also differ among bacterial species. Traditionally, mastitis-causing pathogens have been divided into major and minor pathogens according to the severity of the caused disease, degree of economic loss or infectious features they possess. The most important major pathogens worldwide are *Staphylococcus (S.) aureus* and streptococcal species (Barkema et al., 2006;

Østerås et al., 2006; Bradley et al., 2007; Persson Waller et al., 2009; Vakkamäki et al., 2017). The proportion of minor pathogens, especially of non-aureus staphylococci, has continuously increased (Pitkälä et al., 2004; Vakkamäki et al., 2017), currently representing the most prevalent mastitis-causing organisms in subclinical mastitis and IMI in many countries, for example in Finland, the Netherlands and Germany (Sampimon et al., 2009; Tenhagen et al., 2006; Taponen and Pyörälä, 2009; Vakkamäki et al., 2017). Different factors predispose dairy cows to mastitis, including management, stall type, milking system, breed, herd characteristics and season (Østerås et al., 2006; Lievaart et al., 2007; Hiitö et al., 2017; Taponen et al., 2017).

Identification of the mastitis-causing pathogens enables one to target the treatments appropriately, to avoid unnecessary antimicrobial treatments, and to create the best possible herd level management protocols. Diagnosis of IMI is based on microbial culture of aseptically taken quarter-milk samples (International Dairy Federation, 2011). Cow composite (Reyher and Dohoo, 2011) and bulk tank milk samples (Fox et al., 2005; Rysanek et al., 2009; Barkema et al., 2009) have also been used in mastitis diagnostics, mainly in eradication and surveillance programmes. Conventional culture has been, and still is, the reference (gold standard) method to identify the microbiological cause of IMI (Griffin et al., 1977; Middleton et al., 2017).

The modern dairy industry has elicited a need for more accurate and faster methods to diagnose bovine IMI. DNA-based molecular diagnostic assays, for example different variations of the Polymerase Chain Reaction (PCR), including conventional, real-time and multiplex real-time PCR, have been introduced in bovine mastitis diagnostics since the beginning of the 21st century. Advantages of DNA-based methods are greater objectivity, increased speed and higher sensitivity (Gillespie and Oliver, 2005; Taponen et al., 2009; Koskinen et al., 2009). The ability to detect DNA also from dead and growth-inhibited pathogens may also be considered an advantage in some contexts (Taponen et al., 2009; Bexiga et al., 2011a). Finland was the first country to adopt PCR for routine mastitis diagnostics. Currently, over 80% of all milk samples in Finland are tested only with commercial multiplex real-time PCR assay instead of culture. The method was taken into use relatively quickly by the dairy industry, but it seems to work well in routine use (Vakkamäki et al., 2017). The interpretation of the results has been challenging in the absence of agreed guidelines and lack of comprehensive scientific knowledge. This has been especially true when results have included several different target species.

This dissertation is comprised of a review of bovine mastitis diagnostics, focusing on PCR diagnostics, and four independent studies where multiplex real-time PCR was used in bovine mastitis diagnostics.

2 Review of the literature

2.1 Background in diagnosing bovine intramammary infection (IMI)

Mastitis is one of the major reasons to use antimicrobials in dairy cows (Pol and Ruegg, 2007). Historically, the goal of treatment of mastitis has not necessarily been bacterial cure, but normalization of the milk, to continue delivery of milk of the mastitic cow to the dairy as soon as possible (Barlow, 2011). In countries where veterinary consultation in treatment of mastitis is not obligatory, routine treatment with broad spectrum antimicrobials has been the rule (Barlow, 2011; Oliver and Murinda, 2012; Cha et al., 2016). Use of antibiotics in dairy cows may contribute to an increase in antimicrobial resistance. With current concerns and evidence of the global spread of antimicrobial resistance (WHO, 2018) antimicrobial use should be justified and prudent.

Only a minority of the clinical mastitis cases benefit from antimicrobial treatment, depending on the causative agent (Oliveira and Ruegg, 2014; Ruegg, 2014). Knowledge of the causative agent provides a basis to avoid unnecessary antimicrobial treatments and expensive withdrawal times. With pathogens, where antimicrobial intervention is relevant (Pyörälä, 2009), the treatment can be targeted accordingly with the most suitable narrow-spectrum antimicrobials and adequate length of the course. The on-farm data, consisting of aseptically taken quarter milk samples over a longer period, serve as the basis for the mastitis management and udder health protocols on each farm.

An aseptic milk sample from the mastitic quarter can be submitted for microbiological analysis to diagnose the causal pathogen. In Nordic countries, sampling of quarters with suspected mastitis has been successfully advised for decades (Hiitiö et al., 2017; Vakkamäki et al., 2017). Unfortunately, routine identification of mastitis-causing pathogens from the infected quarter elsewhere has been rare (Ruegg, 2014; Cha et al., 2016).

The first level of identification with culture-based methods is the division of microbes according to Gram-staining, into Gram-negatives or Gram-positives. This is possible even on dairy farms using on-farm culture methods (Lago et al., 2011; Ruegg, 2014; Cha et al., 2016). This level of identification can be used in deciding whether to treat or not to treat clinical mastitis. It has been estimated that 50 – 80% of Gram-negative mastitis cases do not require antimicrobial treatments due to high levels of antimicrobial resistance and spontaneous cure rates (Roberson, 2003; Suojala, 2010; Schukken et al., 2011). Spontaneous cure rates and antimicrobial resistance differ also among Gram-positive pathogens (Barlow, 2011). The ideal protocol is to send a milk sample to a laboratory, where the microbe can be identified at a species or a group of species level and antimicrobial susceptibility can be tested if deemed necessary (Middleton et al., 2017). This level of identification increases the immediate diagnostic costs, but can provide valuable information, especially if the identified pathogen is contagious (Samson et al., 2016; Cha et

al., 2016). Due to the logistic challenges with milk samples and the culture process itself, obtaining the results may take several days (Cha et al., 2016). A long response time delays the start of the treatment and may affect the outcome and further spread of some pathogens, for example *S. aureus* (Barkema et al., 2006; Cha et al., 2016).

2.2. Indicators of inflammation

Milk quality can be evaluated, and suspected mastitis cases confirmed by measuring different indicators of inflammation from milk.

2.2.1. Somatic cell count (SCC)

SCC is the most used and studied indicator of inflammation in milk. In normal milk of a healthy quarter, SCC is clearly under 100 000 cells/ml (Pyörälä, 2003; Schukken et al., 2003), with the cell population comprising mostly macrophages (Rainard and Riollet, 2006). Variation in SCC is caused by stage of lactation, milking, parity, breed and stress in non-infected quarters (Schukken et al., 2003; Bradley and Green, 2005; Olde Riekerink et al., 2007), but inflammation of the mammary quarter results in definite increase of SCC. SCC increases because of an excessive recruitment of neutrophils to the inflamed tissue and milk (Rainard and Riollet, 2006). Some pathogens elicit a greater SCC response than others (Djabri et al., 2002; Simojoki et al., 2011; Schukken et al., 2011). SCC over 200 000 cells/ml indicates a high probability of mastitis (Schepers, 1997; Schukken et al., 2003). In cases where the immune system fails to remove the microbe, a chronic infection follows, maintaining high SCC for a longer period (Schukken et al., 2003). SCC levels can be measured and monitored at quarter-, cow- and herd-level (Schukken et al., 2003).

The California Mastitis Test (CMT) is the most common cow-side method for detecting quarters with increased milk SCC. An advantage of this simple test is that inter-quarter comparison is automatically incorporated into the test, which facilitates the interpretation. In the CMT test, 3 ml of milk and 3 ml of CMT reagent are mixed on a test plate. The test reagent reacts with DNA: the more DNA (from somatic cells) the milk sample contains, the greater the viscosity on the test plate (International Dairy Federation, 2013). Invariably a scoring system on a scale of 1 – 5 is used, where 5 indicates SCC over 5 000 000 cells/ml of milk (International Dairy Federation, 2013). Despite CMT being highly subjective, rough estimates of the SCC can be generated and its value in practice is high (Sargeant et al., 2001). Several automatic methods are available for determination of SCC, such as electro-optical and fluoro-optical devices (Pyörälä, 2003). Automatic milk systems use their own methods for monitoring SCC, such as the Online Cell Counter (OCC, DeLaval, Sweden) (Sørensen et al., 2016).

2.2.2. Other indicators

Other less frequently used indicators of mastitis are also available, including N-acetyl- β -D-glucosaminidase, milk amyloid-A and lactate dehydrogenase (Pyörälä, 2003). The amount of lysosomal enzymes originating from phagocytes or epithelial cells, such as N-acetyl- β -D-glucosaminidase (NAGase), increases during inflammation (Kitchen et al., 1980), reflecting the destruction of mammary tissue. In subclinical mastitis, the overall performance of this kind of indicator has been reported to be inferior compared with SCC (Nyman et al., 2014; Nyman et al., 2016b) for measuring the actual inflammatory response. LDH and NAGase results are more affected by cow factors than SCC in IMI negative cows (Nyman et al., 2014). Concentration of NAGase activity is also associated with the amount of bacterial DNA in milk samples (Kalmus et al., 2013).

NAGase activity in milk varies considerably, especially among healthy cows (Chagunda et al., 2006b). NAGase activity has been reported to be higher in clinical than in subclinical mastitis (Pyörälä, 2003; Chagunda et al., 2006b). Milk NAGase releases 4-methylumbelliferon (4-MU) in an acidic environment, which fluoresces in an alkaline environment. NAGase activity is measured against a known 4-MU concentration and reported as picomoles of 4-MU/min per microliter of milk (Hovinen et al., 2016). The upper limit for normal milk is 1.04 pmol of 4-MU/min per microliter of milk and the upper quantification limit is 24.49 pmol of 4-MU/min per microliter of milk (Hovinen et al., 2016). Taponen et al. (2009) reported an average NAGase activity for clinical mastitic cases of 18.7 pmol of 4-MU/min per microliter of milk (Taponen et al., 2009). Chagunda et al. (2006b) used another modification of the test, reporting a mean for clinical mastitis cases of 69.42 micromoles $\text{min}^{-1} \text{l}^{-1}$. NAGase has been reported to be a reliable indicator at least for some pathogens (Kalmus et al., 2013), but SCC still possesses the best overall ability to identify IMI over lactate dehydrogenase and NAGase (Nyman et al., 2016b).

Electrical conductivity (EC) is the most commonly used method to detect mastitis online and in automatic milking systems (AMS). It is based on the increase of Na^+ and Cl^- in mastitic milk, but it has been reported to be inadequate to detect subclinical or clinical mastitis (Hovinen et al., 2006). Changes in colour of milk may also be detected and this is a commonly used indicator of mastitic milk in visual inspection as well as in AMS detection devices. However, automatic diversion of milk, based on changes in colour or EC, has been reported to be unreliable (Hovinen and Pyörälä, 2011). Biological models to combine the information of different indicators of mastitis for AMS have been introduced (Chagunda et al., 2006a) and continuously developed.

2.3. Microbiological diagnosis of bovine IMI

2.3.1 Milk sampling

2.3.1.1. Aseptic quarter milk sampling

Aseptic quarter-based milk sampling according to international standard methods (Middleton et al., 2017) is a prerequisite for a reliable microbiological result in mastitis diagnostics. The barn environment is a challenge for aseptic procedures. Complete asepsis cannot be achieved but all possible sources of contamination during sampling should be minimized. First, the udder should be brushed from possible loose dirt, hair and bedding material. Several streams of milk are milked on to a test plate and the milk is visually inspected. A pair of disposable gloves is put on. The teat end of the test quarter is cleaned with cotton moistened with 70% alcohol, at least three times or until visibly clean. The cap of the sterile milking tube should be protected, and the tube kept in a nearly horizontal position during sampling to avoid contamination from above. A few centimetres of milk in the bottom of the tube is enough for analysis. Tubes are marked with date and identification of the cow and quarter and stored in a refrigerator until culturing or with preservative tablets if studied with PCR assays.

2.3.1.2. Cow composite sampling

Cow composite samples include milk from all quarters of the cow combined in one test tube. The traditional purpose of composite samples is to determine content of certain milk solids and SCC at the cow-level for dairy herd improvement (DHI), but they might also be used to diagnose subclinical IMI with microbiological tests (Reyher and Dohoo, 2011). Composite samples are usually taken with automatic sampling devices during milking, without any disinfection of the teats. The possibility of environmental and skin contamination is high and samples are prone to carryover phenomena, e.g. microbial contamination from preceding cows due to the function of the sampling devices (Løvendahl and Bjerring, 2006; Mahmmod et al., 2013a; Mahmmod et al., 2014). Composite samples can also be collected following aseptic sampling techniques by collecting milk aseptically from each quarter into the same, single tube. With this method, some level of asepsis can be maintained.

The diagnostic goal of cow composite samples is mainly to screen cows for specific pathogens. This kind of information may be used in eradication programs and surveillance of highly contagious pathogens like *Mycoplasma (M.) bovis* (Fox, 2012). Results from non-aseptic samples have been used in cow level mastitis diagnostics (Cederlöf et al., 2012; Mahmmod et al., 2013b). Definition for true IMI based on these samples has been difficult because, for example, *Streptococcus (Str.) agalactiae* has been shown to be present in the environment (Jorgensen et al., 2016) and *S. aureus* (Barkema et al., 2006) is known for

colonizing the skin of the udder. A large Canadian study, where composite samples were cultured to diagnose subclinical IMI, concluded that sensitivity (Se) and specificity (Sp) of culture were at moderate levels, differed markedly among species and improved when more than one quarter of the cow was infected (Reyher and Dohoo, 2011).

2.3.1.3. Bulk tank milk (BTM) sampling

An indication for microbiological analysis of bulk tank milk is to evaluate milk quality and possibly udder health of the tested herd (Barkema et al., 2009; Rysanek et al., 2009; Katholm et al., 2012). It can be used in surveillance of certain udder pathogens like *M. bovis* and *Str. agalactiae* at herd-level (Fox et al., 2005; Barkema et al., 2009; Katholm et al., 2012). Microbiological analysis of bulk tank can be done with culture or PCR assays with specific selection of target pathogens.

2.3.1.4. Carryover in milk sampling

Carryover is a phenomenon where the present milk sample includes residual milk from previous samples. In AMS, milk samples are collected with built-in meters. Residual milk from a previous cow may remain in the milking unit(s) and cause cross-contamination. Carryover has been reported to occur in parlour milking (Mahmmod et al., 2013a; Mahmmod et al., 2014; Mahmmod et al., 2017) and the sampling devices of automatic milking robots (Løvendahl et al., 2010; Da Silva et al., 2016). It may affect pregnancy-associated glycoprotein (PAG) levels (Da Silva et al., 2016) milk solids like fat, SCC (Løvendahl et al., 2010) and mastitis pathogen results (Mahmmod et al., 2013a; Mahmmod et al., 2014; Mahmmod et al., 2017). Carryover is suspected also in bulk tank milk samples (Nielsen et al., 2015), where residues of milk may be present in taps of the bulk tanks and other sampling equipment.

2.3.2. Conventional bacterial culture (BC)

Bacterial culture (BC), carried out according to the internationally agreed standards (Middleton et al., 2017) is considered the reference method in mastitis diagnostics. BC is based on culturing a milk aliquot from an aseptically taken quarter milk sample on an agar plate and incubating the plate for 24 to 48 hours. Viable microbial cells can be detected as colony forming units (cfu). Representatives of morphologically different colonies, indicating different species, are identified according to standard microbiological schemes (Middleton et al., 2017). If the agar plate contains more than two different species, the sample is discarded as contaminated.

One of the major advantages of BC is that only viable microbial cells are detected. Subsequently, it can be assumed that these viable microbes are also capable of inducing mastitis. No agreed standard for the detection limit of bovine intramammary infection (IMI) is available. Andersen et al. (2010) classified IMI in two separate definitions: 1) milk sample containing an organism of interest with $\geq 1\ 000$ cfu/ml on the test day or 2) the organism of interest isolated in three consecutive weekly samplings at least twice. Dohoo et al. (2011) concluded that a criterion for positive IMI when examining only a single milk sample could be a single colony from a 0.01 ml milk sample (resulting detection limit of 100 cfu/ml), with the exception of CNS, where 2 colonies per 0.01 ml of milk would be a more appropriate limit (Dohoo et al., 2011). In surveys around the world, definitions for positive IMI have varied and often been left unannounced. For example, Bradley et al. (2007) used the most common limit of 100 cfu/ml for positive IMI, Pitkälä et al. (2004) used 500 cfu/ml and Østerås et al. (2006) adjusted the detection limit according to the pathogen.

The definition of a gold standard is 'a perfect reference test'. It can be argued that BC does not meet this requirement because several disadvantages of the method can be recognized. One of the most commonly acknowledged problems of BC is represented by milk samples without microbial growth. This is also true for clinical mastitis cases, where the milk is visibly changed and signs of inflammation may be seen in the affected udder and the cow, in addition to high SCC. In clinical mastitis cases, proportions of negative samples vary between 10 – 27% (Sargeant et al., 1998; Waage et al., 1999; Bradley et al., 2007; Koivula et al., 2007) and in subclinical cases the proportions are even higher (Bradley et al., 2007; Koivula et al., 2007). Several different reasons for culture-negative samples have been proposed. The amount of pathogen in the milk sample may simply be too low, or the infection be of very short duration (Sears and McCarthy, 2003; Walker et al., 2011). Sensitivity of positive detection increases when inoculum size is increased, at least for *S. aureus* (Walker et al., 2010). In addition, bacteria are not homogeneously distributed in samples. Due to the immune response against the invading microbe, mastitic milk contains several growth-inhibitory compounds that may inhibit bacterial growth on agar plates (Rainard and Riollot, 2006). The milk sample may also contain antimicrobial residues if it is collected too soon after antimicrobial treatment in an attempt to confirm bacteriological cure.

BC is also time consuming: accurate identification of the causative pathogen is often only possible after 48 hours of incubation and further diagnostic tests. For some pathogens, like *M. bovis*, correct identification requires special media and incubation times up to 6 – 10 days (Maunsell et al., 2011). In addition to international guidelines for mastitis diagnostics (Middleton et al., 2017), standards including the requirements for the laboratory, quality control and traceable results, are available. ISO 17025 is the main standard for laboratories producing testing and calibration results. Despite standardized procedures in BC, considerable variation in diagnostic procedures and interpretation of the results has been reported (Pitkälä et al., 2005; Karlsmose et al., 2013).

2.3.2.1. *On-farm culture systems*

Several on-farm tests for mastitis diagnostics have been introduced. On-farm culture systems are based on selective agar media or petrifilms. Milk samples are cultured on agars, promoting the growth of microbes possessing certain characteristics. Most common plates target differentiating between Gram-negative and Gram-positive microbes. The Di-Plate (Minnesota Easy Culture System II, University of Minnesota Laboratory for Udder Health, MN) system includes two media, one selective for Gram-negative microbes and one for Gram-positive, hindering the growth of Gram-negatives. A Tri-Plate system includes Gram-negative selective agar (McConkey agar) and a medium for growth of catalase-negative Gram-positive cocci (most commonly streptococci) in addition to the conventional tryptic soy blood agar (Minnesota Easy Culture System, University of Minnesota Laboratory for Udder Health, MN). Viora et al. (2014) used a three-plate test kit (VetoRapid, Vétoquinol, Buckinghamshire, UK), where one-third of the plate is a selective agar for coliforms, one third a modified mannitol salt agar and one third is a modified Edwards agar (Viora et al., 2014).

Royster et al. (2013) compared the Di-Plate and Tri-Plate systems with conventional bacterial culture in identification of mastitis pathogens. They concluded that results from Di-Plate and Tri-Plate were most reliable when used only to diagnose three robust categories of Gram-negative, Gram-positive and no growth (Royster et al., 2014). Viora et al. (2014) reported that test reliability between tri-plate agar and conventional culture varied according to the causal pathogen. McCarron et al. (2009) compared the Di-Plate media with the 3M Petrifilm system in diagnosing clinical mastitis. 3M Petrifilm system included two films, one film selective for aerobic microbes and one for coliforms (McCarron et al., 2009). They concluded that both Bi-Plate and 3M Petrifilm systems performed well in categorizing isolates from clinical mastitis cases in two groups: Gram-positive and Gram-negative. Neither test could reliably determine contaminated samples (McCarron et al., 2009). 3M Petrifilm has been tested for even more robust characterization of bacterial growth or no bacterial growth for low SCC cows before dry-off (Cameron et al., 2013). Reasonable sensitivity (85.2%) for the 3M Petrifilm system was reported (Cameron et al., 2013).

On-farm culture has been reported to have substantial benefits on those farms where clinical mastitis has been based on only empirical, sign-based treatments by the farmer (Lago et al., 2011). Benefits have been reported also if blanket dry cow therapy has been changed to selective dry cow therapy (Lago et al., 2011; Cameron et al., 2014). Lago et al. (2011) reported reduction of intramammary antibiotics by half after implementing an on-farm culture system, without significant difference in clinical cure.

2.3.3. Detection of pathogen DNA with polymerase chain reaction (PCR) and its variations

According to Gillespie and Oliver (2005) one of the greatest single advantages of DNA-based molecular methods is the focus on identification of actual nucleic acid compositions of the microbial genome instead of only the phenotypic expression of the products that those nucleic acids encode. Several different DNA-based molecular methods exist in modern microbiology (Tang, 2013) and more assays are constantly developed. The most common DNA-based molecular method to date in detecting pathogen DNA from pathogens involved with bovine IMI is the PCR and its variants.

PCR is a very delicate *in vitro* molecular technology, where multiple copies of DNA segments of interest are generated within a few hours using polymerase enzymes. It has been widely used in detecting disease-causing organisms and has become a standard method in diagnostic microbiology (Tang, 2013). PCR was developed and introduced by Mullis et al. (1986). The components of a PCR reaction include purified and extracted target-DNA (e.g. the sample or template), polymerase enzyme, primers, deoxynucleotide triphosphates (dNTP) and buffer solution with optimized pH and concentrations of various substances including enzyme cofactor magnesium (Mullis et al., 1986).

2.3.3.2. Polymerase enzyme

Polymerase enzymes originate from thermophilic bacteria (Innis et al., 1990; Haki and Rakshit, 2003). These bacteria have adapted to extreme temperatures with adapted cell membranes, positive supercoils in their DNA and by producing thermostable proteins (Haki and Rakshit, 2003), among other characteristics. One of the first and most frequently used polymerase enzymes is *Taq* polymerase from the bacterium *Thermus aquaticus* (Haki and Rakshit, 2003). The purpose of the polymerase enzyme is to serve as the catalyst for the elongation phase (Innis et al., 1990) of the PCR cycle. The polymerase enzyme attaches dNTP according to the template base order.

2.3.3.3. Primers

Primers are synthetic oligonucleotide sequences, which precede the desired target DNA sequence. A pair of primers attach to each complementary strand of DNA marking the initiation points for amplification (Tang, 2013). Without a primer, the polymerase enzyme cannot work because it requires double-stranded DNA (Tang, 2013).

Primers are relatively short (10 – 30 nucleotides) and of 50 – 60% guanine-cytosine (G+C) composition (Innis et al., 1990). Successful design of primers is a prerequisite for optimal PCR reaction. The length and sequence of the primer defines the melting, and

consequently the annealing temperature (Tang, 2013), depending on the number of hydrogen bonds. Various different softwares are available for primer design (Pereira et al., 2008). Concentration of primers affects the accuracy of the PCR reaction: if primer concentration is too high, primers may attach to each other, making primer-dimers and consequently lowering the amount of detected target-DNA (Innis et al., 1990).

2.3.3.4. PCR cycle

The PCR cycle consists of three process steps: denaturation, annealing and extension. In denaturation, the coiled DNA strands are separated at a temperature of 90 – 95 °C (Mullis et al., 1986). If the denaturation temperature is too high, DNA may break and if it is too low, the hydrogen bonds between the strands do not open. The annealing phase is carried out at a lower, 55 – 75 °C, temperature, allowing the primers to anneal correctly. The temperature affects the accuracy and degree of attachment. In the final extension phase, polymerase enzyme catalyses the synthesis of the target DNA copy from the dNTP. With this complementary base-pairing a new strand is formed (Mullis et al., 1986).

The PCR cycles are induced in thermal cyclers, where temperatures and times of cycle steps can be more effectively maintained and changed, compared with the first versions in water baths (Innis et al., 1990). After the first three cycles, two copies of the target DNA and six fragments with variable lengths are produced. After 20 cycles the number of target DNA compared with the variable length fragments is over 1000-fold. When conditions are optimal, the number of target DNA strands is doubled with every cycle. The maximum number of cycles is 40.0. Cycles over 40.0 produce nonspecific background products of no value (Mullis et al., 1986; Innis et al., 1990) and with suboptimal reproducibility.

2.3.3.5. Detection of PCR products

DNA sequences must be identified after amplification. DNA is a negatively charged molecule (Nelson et al., 2005) with a natural tendency to move towards a positive pole when exposed to an electric current. In gel electrophoresis, DNA products are subjected to electrical current in an agarose matrix. Fragments line up according to their size as smaller products move faster than larger ones in the gel matrix. Visualization of the patterns is possible with ethidium bromide (Tang, 2013). Identification of species is done by comparing obtained patterns with reference patterns.

2.3.3.6. Real-time PCR

In real-time PCR, the PCR product can be detected during the amplification process (Tang, 2013) and detection of PCR products is enabled by primers with fluorescence-

labelled probes. These labelled primers are termed oligos (Klein, 2002). Fluorescence can be detected when the probe detaches or is hybridized (Klein, 2002). The amount of fluorescence increases in direct proportion with the target DNA (Tang, 2013). To date, the most used PCR chemistries are SYBR Green and TaqMan. With SYBR Green, the fluorescence is emitted when binding to the double-stranded DNA occurs and with TaqMan, fluorescence is emitted only when the correct product has been formed (Tang, 2013). Fluorescence is detected with automated optic devices.

Measurement of fluorescence is carried out in every cycle, enabling quantitative features of real-time PCR (Pereira et al., 2008). Real-time PCR can be considered a semi-quantitative method because the number of products is not exact, but the result of an external standard curve (Pereira et al., 2008; Tang, 2013). The cycle threshold value (Ct) is the number of cycles needed to obtain a predetermined level of fluorescence. The greater the amount of target DNA in a sample, the earlier the detection threshold is achieved (Tang, 2013). Advantages of real-time PCR over conventional PCR are the higher precision and technical sensitivity (<5 microbial copies) (Klein, 2002) and lower turn-around times (Phuektes et al., 2001; Klein, 2002; Pereira et al., 2008; Koskinen et al., 2009). Post PCR handling can be avoided, minimizing the risk of cross-contamination in the laboratory (Pereira et al., 2008). Edwards and Gibbs (1994) concluded that most real-time PCR assays showed high precision and reproducibility, but the accuracy of the data depended on sample preparation, quality of the standard and the chosen target gene (Edwards and Gibbs, 1994).

2.3.3.7. Multiplex real-time PCR

Multiplex real-time PCR reactions include oligos for several different targets. Primers for multiplex real-time PCR must be designed to be of unique sizes (Tang, 2013) and similar kinetics, otherwise they not able to function (Edwards and Gibbs, 1994; Tang, 2013). With each additional primer, the possibility of nonspecific products increases (Edwards and Gibbs, 1994). Reactions are adjusted according to the primers, target regions, relative size of the target DNA fragments, temperature and other factors suitable for multiplex amplification (Edwards and Gibbs, 1994; Koskinen et al., 2009). If multiplex real-time PCR is properly optimized, the reliability of the data increases and turn-around time and costs are lower compared with conventional and one-species real-time PCR (Edwards and Gibbs, 1994).

No single PCR protocol is suitable for all situations. In addition to applications mentioned here, several others are available (Tang, 2013) and the choice of method is determined by the purpose. Different PCR assays need optimization for polymerase enzyme, dNTP and magnesium (enzyme cofactor) concentrations and other reaction components depending on the purpose (Innis et al., 1990). The primer and oligo designs are critical to achieve maximum amplification efficiency. Successfully implemented primers are usually protected with patents in commercial PCR assays. Most common problems with PCR are too low amount or no amplified product at all, mispriming and misextension of the

primers, formation of primer-dimers competing for amplification with target product and mutations in target sequences (Innis et al., 1990). In addition, in multiplex real-time PCR, annealing conditions for several oligos may not be optimal, increasing the chance of errors during the cycle. With PCR applications, the only biological data are the detected target DNA fragments.

2.3.4. Development of the PCR assay for bovine mastitis diagnostics

Different applications of PCR in bovine mastitis diagnostics have been introduced since the beginning of the 21st century (Riffon et al., 2001; Phuektes et al., 2001; Gillespie and Oliver, 2005; Graber et al., 2007; Koskinen et al., 2009). The joint objective for development of a DNA-based molecular diagnostic method was to have a fast test of high sensitivity (Se) and specificity (Sp), providing reliable and objective results.

The first two PCR tests designed to detect udder pathogens were a conventional PCR by Riffon et al. (2001) and a multiplex PCR assay by Phuektes et al. (2001). Primers designed for 23S and 16S rRNA spacer regions were used in both studies. Riffon et al. (2001) tested primers for *Escherichia (E.) coli*, *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, *Str. parauberis* and *Str. uberis*. Electrophoresis was used to detect the amplified PCR products. Time used in the test was 4.5 - 6 h depending on whether a pre-PCR enzymatic lysis step was included or not. Deleting the pre-PCR enzymatic lysis step decreased the time and expenses but lowered the sensitivity of the test from 3.13×10^3 cfu/ml to 5×10^3 cfu/ml (Riffon et al., 2001). Phuektes and al. (2001) presented a multiplex PCR assay for simultaneous detection of *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis*. They reported detection limits for multiplex PCR from 10^3 and 10^4 cfu/ml depending on the species and DNA extraction method (Phuektes et al., 2001). Sensitivity of 1 cfu/ml was obtained with overnight enrichment protocol. The multiplex PCR was reported to be significantly more sensitive than culture for detection of *S. aureus* and *Str. uberis*, but no significant differences in sensitivities between PCR and culture for the detection of *Str. agalactiae* and *Str. dysgalactiae* were established (Phuektes et al., 2001).

Gillespie and Oliver (2005) introduced a multiplex real-time PCR assay to detect *S. aureus*, *Str. uberis*, and *Str. agalactiae* simultaneously with primers other than 16S and 23S rRNA. The sensitivity of this assay to correctly identify *S. aureus*, *Str. agalactiae*, and *Str. uberis* directly from milk was 95.5% and specificity was 99.6% (Gillespie and Oliver, 2005), considering BC as the gold standard.

Graber et al. (2007) used real-time quantitative PCR (qPCR) in detection of only *S. aureus* by targeting the *nuc* gene (Graber et al., 2007) with analytical sensitivity of 1.15×10^3 cfu/ml. They reported that qPCR was never negative for *S. aureus* when the culture result was positive and analytical sensitivity was reported over 500 times higher than in standard bacteriology (Graber et al., 2007). Correlation between the qPCR results and cfu/ml was high (0.93) (Graber et al., 2007). This qPCR was applied also in a field study to

identify chronic *S. aureus* infections (Studer et al., 2008), where a clear synchrony in the shedding curves between qPCR and cfu/ml was reported. Botaro et al. (2013) introduced another qPCR assay targeting *S. aureus* with different quantification chemistry (SYBR GREEN instead of TaqMan) reaching detection limits of 1.04×10^1 cfu/ml (Botaro et al., 2013).

The first commercial multiplex real-time PCR assay for mastitis diagnostics (PathoProof™ Complete-12 Kit; Thermo Fisher Scientific, former Finnzymes Oy, Finland) was introduced by Koskinen et al. (2009). The test-kit included all the needed reagents for DNA extraction and patented primer-mixes and solutions for the PCR. The assay was designed to identify staphylococcal beta-lactamase gene (*blaZ*) and eleven of the most common udder pathogens (*Corynebacterium (C.) bovis*, *Enterococcus faecalis/faecium*, *Escherichia (E.) coli*, *Klebsiella oxytoca/pneumoniae*, *Serratia (S.) marcescens*, *Staphylococcus (S.) aureus*, *Staphylococcus* spp., *Streptococcus (Str.) agalactiae*, *Str. dysgalactiae*, *Str. uberis* and *Peptoniphilus (P.) indolicus* and/or *Trueperella (T.) pyogenes*) to species or a group level directly from milk, without enrichment protocols. The group level identification is based on oligos designed for whole pathogen group (like staphylococci, including species and subspecies). Specific additional oligos were designed for certain species, like *S. aureus*, to separate them from the group. The result *Staphylococcus* spp. in PCR results from PathoProof™ thus refers to non-aureus staphylococci. The kit included an enzymatic lysis step to disrupt the cell walls of bacteria to the extraction protocol, as well as spin column-based DNA purification and elution steps. The results were promising as the analytical Se and Sp of the assay from spiked samples was 100% for all isolates originating from bovine mastitis and the test was completed in four hours (Koskinen et al., 2009).

Since then, several kits of PathoProof™ Mastitis PCR Assay, including different panels of pathogen species, have been launched. The panel currently in routine use in Finland is Thermo Scientific™ PathoProof™ Complete-16 Kit, which has replaced the previous Complete-12 kit. Complete-16 Kit includes four additional oligos (*Mycoplasma* spp., *M. bovis*, *Prototheca* spp. and yeasts) in addition to oligos mentioned above. Dairy producers send milk samples with preservatives to laboratories via milk trucks or mail and receive the answer usually in 24 hours. Some laboratories send the result of a bacterial DNA finding as Ct values, some laboratories provide the result as high (+++), moderate (++) or low (+) amount of detected target DNA, based on Ct ranges provided by the manufacturer. To my knowledge, only one other commercial multiplex real-time PCR assay, Mastit 4 (DNA Diagnostics A/S, Risskov, Denmark), is available to date, including also several different panels with slight differences in the target pathogen spectrum.

2.4. Multiplex real-time PCR in bovine mastitis diagnostics

Studies on the performance and detection capacity of commercial multiplex real-time PCR assays have been published since 2009, often comparing the PCR results with BC results. These studies comprise results from quarter-based milk samples (Koskinen et al.,

2010; Spittel and Hoedemaker, 2012; Keane et al., 2013; Steele et al., 2017), cow composite samples (Cederlöf et al., 2012; Mahmmod et al., 2013b; Nyman et al., 2016a) and bulk tank milk samples (Soltau et al., 2017). Some studies where multiplex real-time PCR has been the only diagnostic method are also available from quarter sampling (Vakkamäki et al., 2017), cow composite sampling (Timonen et al., 2017) and from bulk tank milk (BTM) sampling (Katholm et al., 2012).

2.4.1. Performance and detection capacity of multiplex real-time PCR

The first field study comparing a commercial multiplex real-time PCR assay (PathoProof™ Complete-12 Kit) with conventional culture included a set of aseptically taken quarter milk samples from healthy, subclinical and clinical cases from Finland and The Netherlands (Koskinen et al., 2010). Pathogens were identified and detected from all types of mastitis more often with PCR compared with BC (Ct cut-off 37.0) (Table 1). Similar results were obtained in Ireland, where clinical mastitis samples from Irish milk-recording dairy herds were examined with BC and with the same PCR assay and cut-off (Keane et al., 2013) (Table 1). In a German study carried out with a comparable PCR assay and test panel, quarter milk samples from healthy cows with varying SCC counts were examined (Spittel and Hoedemaker, 2012). They reported that PCR detected target DNA over twice as often compared with BC, where only 32% of samples were positive in culture. A low percentage of positive BC samples was reported to be in line with previous German studies (Spittel and Hoedemaker, 2012), though. Vakkamäki et al. (2017) presented data from over 240 000 quarter milk samples studied only with multiplex real-time PCR assay from dairy cows with suspected mastitis, thus including subclinical and clinical mastitis. They reported that 88% of the samples included DNA of at least one target pathogen (Vakkamäki et al., 2017).

Table 1. Proportion of samples positive in multiplex real-time PCR and culture (BC) in studies using aseptically taken quarter milk samples. PathoProof™ Complete-12 Kit was used in all studies except Vakkamäki et al. (2017) in which Complete-16 Kit was also used for a subset of samples.

	N, samples	Type of mastitis	Proportion of PCR+ samples	Proportion of BC+ samples
<i>Spittel and Hoedemaker, 2012</i>	681	subclinical	71 %	32 %
<i>Koskinen et al. 2010</i>	780	clinical	89 %	77 %
<i>Koskinen et al. 2010</i>	46	subclinical	91 %	83 %
<i>Koskinen et al. 2010</i>	132	healthy	28 %	17 %
<i>Keane et al. 2013</i>	141	clinical	92 %	70 %
<i>Vakkamäki et al. 2017</i>	240067	variable	88 %	n/a

Se and Sp for multiplex real-time PCR have been calculated using various methods (Table 2). Steele et al. (2016) selected a subset from quarter-based, BC positive samples for PCR testing, representing quarters with a current or prior *Str. uberis* or *S. aureus* infection. Compared with culture, PCR performed better in detecting *S. aureus* than *Str. uberis* (Table 2) (Steele et al., 2017). Agreement between BC and PCR was 74% for *S. uberis* and 96% for *S. aureus* (Steele et al., 2017).

Table 2. Sensitivities (Se) and specificities (Sp) for multiplex real-time PCR and bacterial culture (BC) in detecting different target pathogens from milk samples. Detailed description of the samples and methods are presented in the last column.

	Target	Se PCR	Sp PCR	Se BC	Sp BC	Other information
Steele et al. 2016 ¹	<i>S. aureus</i>	96.4	99.7	n/a	n/a	Quarter based samples. Data studied with PCR comprised of a subset from BC+ samples. Ct cut-off 37.0. Se and Sp calculated BC as gold standard.
	<i>Str. uberis</i>	86.8	87.7	n/a	n/a	
Mahmmod et al. 2013b ¹	<i>S. aureus</i>	91	99	53	89	Cow composite samples from lactating cows. Ct cut-off 39.0. Se and Sp calculated with Latent Class Analysis.
Cederlöf et al. 2012 ¹	<i>S. aureus</i>	61-93	99-93	94-78	90-97	Cow composite samples from cows at dry-off. Ct cut-offs varying from 32 - 39.0. Most optimal Ct cut-off was concluded 37.0. Se and Sp calculated with Latent Class Analysis.
Nyman et al. 2016a ¹	<i>S. aureus</i>	91	96	65	90	Lactating cows. Cow composite samples were used for PCR and quarter based for BC. Se and Sp calculated with Bayesian Latent Class Analysis.
	<i>Str. dysgalactiae</i>	80	98	75	98	
	<i>Str. uberis</i>	95	98	75	98	
	NAS ²	75	85	60	80	

¹ PathoProofTM Mastitis Complete-12 Kit ² Non-aureus staphylococci

Se and Sp of PCR against culture have also been studied using cow composite milk samples (Cederlöf et al., 2012; Paradis et al., 2012a; Mahmmod et al., 2013b; Nyman et al., 2016a). Mahmmod et al. (2013b) compared the test characteristics of PathoProofTM Mastitis PCR Assay, BC and CMT in diagnosing *S. aureus*. They examined 609 routine DHI samples of six herds with parlour milking. They estimated Se and Sp of PCR and BC in diagnosing *S. aureus* using latent class analysis (LCA), where PCR outperformed BC (Mahmmod et al., 2013b) (Table 2). Cederlöf et al. (2012) studied also the diagnostic characteristics of the same PCR assay and BC in diagnosing *S. aureus* from composite samples, but instead of lactating cows they tested cows at dry-off. LCA was used to estimate Se and Sp of PCR with different cut-offs for PCR. They concluded that the most optimal cut-off for PCR was 37.0 (Cederlöf et al., 2012). Nyman et al. (2016a) compared PCR results of cow composite samples with culture results from aseptically taken quarter milk samples, estimating Se and Sp for several different target pathogens (Table 2). The aseptic quarter milk samples of all quarters of the cow were collected over three consecutive days and PCR samples only on day two. They reported that Se of PCR was higher than that of BC for all investigated bacteria if only parallel samples of the second day were tested. If the three consecutive samples were included in BC, Se of BC was numerically higher compared with PCR for only *S. aureus*. They concluded that using non-aseptically collected milk samples increases Se (although not always significantly), while Sp is kept on a similar level or lower (sometimes significantly lower), compared with BC of aseptically collected milk samples (Nyman et al., 2016a).

Paradis et al. (2012) used a different, non-commercial multiplex real-time PCR assay, detecting *S. aureus*, *Str. uberis*, *E. coli* and *Str. agalactiae*, and assessed the samples according to severity of mastitis. They concluded that Se of PCR was higher than Se of BC in samples from healthy quarters, but lower than BC in clinical mastitis samples using latent class analysis (Paradis et al., 2012). They also reported that detection capacity of PCR was reduced for *E. coli* and *Str. agalactiae* from nonclinical samples, when the age of sample increased (Paradis et al., 2012). Due to the differences in methodologies, these results are not applicable to results obtained with other PCR assays. Another commercial multiplex real-time PCR assay with oligos for *S. aureus*, *Str. agalactiae*, *Str. uberis* and *M. bovis* (Mastit 4B) was used by Timonen et al. (2017), where routine DHI samples were examined to identify the within herd prevalence of *M. bovis* in one large Estonian herd. They detected DNA of at least one of the target pathogens in 38% of cow composite samples (Timonen et al., 2017).

Multiplex real-time PCR has been used also to study BTM samples. Katholm et al. (2012) used a PathoProofTM Complete-12 Kit in defining the quality of Danish BTM samples of over 4 200 herds. They concluded that low Ct values (defined as <32.0) for *S. aureus*, *Str. uberis*, *Str. dysgalactiae* and *Str. agalactiae* correlated with high BTM SCC, with the strongest correlation for *S. aureus* and *S. uberis* (Katholm et al., 2012). They also stated that PCR could be used similarly as culture methods in monitoring BTM samples, especially for *Str. agalactiae* (Katholm et al., 2012). Four different species were detected in 90% of the results (Katholm et al., 2012). This indicates that BTM samples include DNA

from several different sources in addition to one or more possibly infected quarters. In addition, this study showed that 95% of samples were positive for *Str. uberis* DNA and 91% for *S. aureus* DNA. In addition to being major mastitis pathogens, *Str. uberis* is present, for example, in cow manure (Klaas and Zadoks, 2017) and *S. aureus* is a common inhabitant of the skin (Barkema et al., 2006). Despite the test panel used, BTM samples leave the origin of the detected pathogen completely open. Detecting DNA of rare, highly contagious major pathogens, like *M. bovis* in Nordic countries, the DNA detection from BTM may be of more diagnostic value and lead to cow level screening for positive carrier animals.

Justice-Allen et al. (2011) compared capacity of PCR for detection of *M. bovis* with BC from BTM samples. They used SYBR Green chemistry in their PCR assay. Despite numerically higher Se for PCR (78%) compared with BC (70%), the difference was not significant. The agreement between the test methods was nearly 80% (Justice-Allen et al., 2011). In a recent study by Soltau et al. (2017) BTM samples were used to estimate the apparent within herd prevalence for major pathogens (*S. aureus*, *Str. uberis*, *Str. agalactiae* and *Str. dysgalactiae*) with a commercial multiplex real-time PCR (Mastit 4B) compared with BC. PCR was concluded to outperform BC on herd level sensitivity (Soltau et al., 2017). With a probability threshold of 90%, one positive BTM sample per herd for the pathogen indicated a cow level prevalence of 28% for *S. aureus*, 9% for *Str. dysgalactiae* and 14% for *Str. uberis* (Soltau et al., 2017).

2.4.2. Number of detected species per sample

Some studies on milk microbiota have revealed a great diversity of microbial species in mastitic milk samples, but their clinical relevance remains completely open (Oikonomou et al., 2012). The current concept is that intramammary infection is caused by one or a maximum two species (Middleton et al., 2017; Rainard, 2017) despite the debated hypotheses about mastitis being related to dysbiosis of the mammary gland (Addis et al., 2016). Consequently, samples with more than two species are considered to be contaminated in BC (Middleton et al., 2017). No agreed consensus for contamination for multiplex real-time PCR results is available and currently all detected target pathogens are reported to the client.

The proportion of positive PCR samples containing more than two target pathogens has varied according to the country and type of mastitis (Koskinen et al., 2010; Spittel and Hoedemaker, 2012; Keane et al., 2013; Vakkamäki et al., 2017), but with the contamination definition of BC, most of the samples have been of good quality (Table 3). In Finland, with data covering over 240 000 routine quarter milk samples taken mostly by farmers, 74.2% of the samples contained only one or two pathogens (Table 3) and 13.4% of samples included DNA of three or more target pathogens (Vakkamäki et al., 2017). The most prevalent target pathogens from these samples were *Staphylococcus* spp. and *C. bovis*. *T. pyogenes*/*P. indolicus* was also a frequent finding in multispecies samples (Vakkamäki et al., 2017). Koskinen et al. (2010) reported notably higher figures in their study, where

aseptic milk samples were collected in two countries. Proportion of positive PCR samples with two or more species was 24.7% (137/554) compared to culture results where 11.1% (65/540) had two or more species (Koskinen et al., 2010). Bexiga et al. (2011a) reported a 4-6% prevalence of multispecies samples. Only three samples (2.0%) included three target pathogens in a study from Ireland (Keane et al., 2013) (Table 3).

Table 3. Percentage of samples where no (0) target pathogen DNA was detected and samples containing target DNA of 1 - 5 different species per sample of aseptically taken quarter samples, studied with real-time PCR (PathoProofTM Complete-12 or Complete-16 Kit).

	N, total	Mastitis	Number of species per sample (%)					≥5
			0	1	2	3	4	
<i>Koskinen et al. 2010</i> ¹	132	healthy	72	22.7	4.5	0.8		
<i>Spittel et al. 2012</i> ¹	681	subclinical	29.4	39.8	24.2	5.5	0.7	0.3
<i>Koskinen et al. 2010</i> ¹	46	subclinical	8.7	58.7	21.7	10.8		
<i>Koskinen et al. 2010</i> ¹	780	clinical	11.4	40.7	30.3	17.6		
<i>Keane et al. 2013</i> ¹	141	clinical	8	63	27	2		
<i>Vakkamäki et al. 2017</i> ^{1,2}	240 067	variable	12.4	49	25.2	9.2	2.9	1.3

¹ PathoProofTM Mastitis Complete-12 Kit ² PathoProofTM Mastitis Complete-16 Kit

Timonen et al. (2017) reported that 61% of cow composite samples contained only one species, 31% contained two and 8% three species, but their test kit included only four major pathogens: *M. bovis*, *Str. agalactiae*, *S. aureus* and *Str. uberis*. They used True-Test automatic sampling device (Timonen A., personal communication 11/2017). If kits with broader spectrum were to have been used, the numbers would probably have been much higher for the multispecies samples. In BTM samples, 90% of the samples included four pathogens with a Ct cut-off of 40.0 (Katholm et al., 2012).

2.4.3. Dominance

The semi-quantitative feature of the multiplex real-time PCR enables reporting a possible dominance of a particular pathogen over the others. Vakkamäki et al. (2017) reported that in 25.2% of the PCR positive samples, two species were detected. Dominance (>90% of the total target DNA in the sample) was reported for 66.2% of the double-species samples. The most common dominant species was *Staphylococcus* spp. (non-aureus staphylococci, NAS) (40.2% of samples), followed by *Str. dysgalactiae* (16.7%), *S. aureus* (14.4%) and *Str. uberis* (10.0%) (Vakkamäki et al., 2017). Species accompanying most often the dominant species were *C. bovis*, *Staphylococcus* spp., yeasts, *T. pyogenes*/*P. indolicus*, and *S. aureus*. In contrast *S. dysgalactiae* and *S. uberis* were the most prevalent second pathogens in Irish milk samples (Keane et al., 2013).

2.4.4. Prevalence of different species

Some mastitis surveys have used PCR to study the microbiological etiology of mastitis. Prevalence of the most common pathogens reflects the situation of the country and does not essentially differ from earlier studies that used conventional BC. Reported prevalence varies also according to definitions used: most studies report the proportion of species out of single species samples or out of the total number of positive samples. In a Finnish study by Vakkamäki et al. (2017), the most common pathogens were *Staphylococcus* spp. (43%) and *S. aureus* (21%) in samples where only one species was present. Mean Ct values were 31.5 for *Staphylococcus* spp. and 28.0 for *S. aureus* (Vakkamäki et al., 2017). In Germany, Spittel and Hoedemaker (2012) reported *C. bovis* (45.2%) and *Staphylococcus* spp. (41.3%) as the most common bacteria among single bacteria detections, *S. aureus* being present in 16.9% of those samples. Among all PCR positive samples, the proportion of *Staphylococcus* spp. findings was 34.5%, *Coryneforms* 47.7% and *S. aureus* 9.5%. In the Irish study by Keane et al. (2013) *S. aureus* presented 37%, *Str. uberis* 25% and *E. coli* 18% of the samples containing only one pathogen. The prevalence of *Staphylococcus* spp. was low (4%) (Keane et al., 2013).

Katholm et al. (2012) studied Danish BTM samples with PCR using the highest possible Ct cut-off, 40.0 (PathoProof™ Complete-12 Kit). DNA of *Staphylococcus* spp. was detected from every sample, DNA of *Str. uberis* was detected in 95%, *S. aureus* in 91% and *C. bovis* in 90% of the samples (Katholm et al., 2012). *Str. agalactiae* was identified from 7% of samples.

2.4.5. Culture negative samples examined with PCR

One of the first studies comparing the detection capacity of PCR and conventional culture aimed to investigate culture negative samples with multiplex real-time PCR (Taponen et al., 2009). Of the 79 culture negative milk samples, originating from clinical mastitis cases, 43% were positive in PCR, all containing common mastitis pathogens. Ct values were reported to vary between of 22.2 - 36.7 in positive samples, indicating 2×10^3 to 10^7 genome copies per ml of milk (Taponen et al., 2009). Koskinen et al. (2010) reported that 76% of culture negative (detection limit in BC 100 cfu/ml) samples originating from clinical and subclinical samples were positive for at least some target pathogen DNA in PCR, using the same assay as Taponen et al. (2009). Bexiga et al. (2011a) tested samples with SCC over 500 000 cells/ml with either culture negative or culture positive for single minor pathogen isolates with PCR. From no-growth samples, 47% was PCR positive. In a German study, target DNA was detected in 57.8% of BC negative samples (Spittel and Hoedemaker, 2012). In Ireland, 79% of the culture negative samples were PCR positive (Keane et al., 2013). Culture negative PCR positive results have mostly contained CNS (Taponen et al., 2009; Koskinen et al., 2010; Bexiga et al., 2011a; Spittel and Hoedemaker, 2012) and *C. bovis* (Koskinen et al., 2010; Bexiga et al., 2011a; Spittel and Hoedemaker, 2012) but high percentages of *Str. uberis* (Taponen et al., 2009; Spittel and Hoedemaker,

2012; Keane et al., 2013) and *Str. dysgalactiae* were also reported. The BC-/PCR+ results have also included several major pathogens, like *S. aureus* (Taponen et al., 2009; Koskinen et al., 2010; Spittel and Hoedemaker, 2012) and *Str. agalactiae* (Koskinen et al., 2010) and *E. coli* (Bexiga et al., 2011a). Keane et al. (2013) reported that the amount of DNA detected with PCR was lower in culture negative than in culture positive samples and stated a need for further studies to establish the role of bacterial quantification for defining clinically relevant PCR results. Based on knowledge to date, quantification of bacterial DNA and its relation to clinical severity of mastitis needs further studies.

2.4.6. PCR negative samples positive in culture

PCR negative results, with culture positive identifications are rare, ranging from 2.0% (Keane et al., 2013) to 5.6% (Koskinen et al., 2010) and 6.8% (Spittel and Hoedemaker, 2012). In a study by Koskinen et al. (2010), 93.0% of the microbes cultured from PCR negative samples were not included in the test panel of the PCR assay. Spittel and Hoedemaker (2012) also cultured microbes belonging to the test panels, including *Staphylococcus* spp., from PCR negative samples. They listed several reasons for PCR negative samples: BC positive samples like bacterial number below the PCR detection limit, polymorphism in the PCR target region or contaminated culture plate. Bexiga et al. (2011a) reported one case where PCR targeted *S. aureus* and BC identification result was for *S. intermedius*. In this case, the authors suggested that the misclassification was more likely an error of the biochemical test in phenotypic identification instead of PCR (Bexiga et al., 2011a). Keane et al. (2013) reported that distribution of PCR targets did not differ between results obtained from culture negative and culture positive samples. The exception was *S. aureus*, which was detected more frequently in culture positive samples than in culture negative samples (Keane et al., 2013).

2.4.7. Beta-lactamase gene (*blaZ*)

The presence of the staphylococcal *blaZ* gene, encoding resistance to beta-lactam antibiotics, is automatically reported in PCR results when it is detected in the milk sample (PathoProof™ Complete-12 Kit and Complete-16 Kit; Mastit-4 Tests M4D, M4BD and M4BDF). The origin of *blaZ* detected from a sample cannot be determined or linked to a specific target pathogen. Pitkälä et al. (2007) studied beta-lactamase production from a total of 175 staphylococcal isolates, originating from bovine mastitis samples and dogs. They tested five commercial beta-lactamase tests, the clover leaf test and PathoProof™ Complete-12 Kit. They concluded that PCR was the most reliable test compared with the reference test (Pitkälä et al., 2007). Keane et al. (2013) used the same PCR assay and reported of 93% agreement between *blaZ* and phenotypic tests for all staphylococci in the study comprising quarter samples from clinical mastitis cases.

Spittel and Hoedemaker (2012) reported 37.0% of CNS and 27.7% of *S. aureus* to possess *blaZ*, which was higher than those reported before in German surveys, which had used conventional methods (Spittel and Hoedemaker, 2012). They did not confirm the PCR results with any conventional methods. A study from Ireland with 40.0% of *blaZ* positive staphylococci reported that the obtained figures were in agreement with previous reports, relying on conventional methods (Keane et al., 2013). In BTM samples, *BlaZ* was detected from 78.0% of the samples (Katholm et al., 2012).

3 Aims

The aim of this research was to provide knowledge to improve the interpretation of bovine mastitis diagnostics done with multiplex real-time PCR by

- 1) comparing results of a commercial multiplex real-time PCR assay and BC from natural and experimental IMI and
- 2) evaluating how the quarter milk sampling technique affects the multiplex real-time PCR results.

4 Materials and Methods

4.1. Study designs and data

This thesis comprises results from four independent studies (I-IV). In studies I and IV, microbiological results from mastitic milk samples analyzed with multiplex real-time PCR (later referred only as PCR) were compared with results from the current reference method of conventional bacterial culture (BC). In studies II and III, the effect of two different milk sampling methods on PCR results were evaluated.

The data for study I derived from 294 routine quarter milk samples, collected by farmers and local veterinarians and sent to the regional laboratory of the largest Finnish dairy processor (Valio Oy, Lapinlahti, Finland) between October 2010 and January 2011. Samples originated from naturally occurring, subclinical or clinical mastitis cases during the defined time period. Samples were included to the study in the order of arrival and no selective measures were carried out. The study was a cross-sectional study, analysing data collected routinely at a defined time.

Data for study II were collected in Estonia during two separate sampling visits in November 2012 and in June 2013. A total of 113 quarters from 53 cows of two large Estonian dairy herds were sampled with two different techniques: conventional aseptic milk sampling and experimental needle sampling. Cows were initially enrolled in the study based on cow composite SCC values of >200 000 cells/ml in the most recent DHI results. Ninety-nine quarters included in the study had high SCC according to CMT tests on site (CMT >2). Twenty-two quarters out of the total 113 had CMT of one, and were sampled to represent healthy quarters. Parity of the cows and average milk production of the herds were recorded. The farms belonged to client herds of the Large Animal Clinic of the Estonian University of Life Sciences. The study was an experimental study to validate our two hypothesis that avoiding the teat orifice and teat duct completely would reduce the number of clinically irrelevant potential contaminants in the milk sample. Experimental sampling procedure was approved by the Commission of Animal Trials at the Estonian Ministry of Agriculture (No 7.2-11/1). Samples were collected by the authors.

Data for study III comprised quarter milk samples collected on 44 commercial dairy farms belonging to the practice area of the Production Animal Hospital of the Faculty of the Veterinary Medicine (University of Helsinki), between March 2011 and April 2013. Cows with mastitis suspected or detected by the farm staff were sampled during farm visits in the conventional aseptic manner and experimentally with a sterile teat cannula. Each additional cannula sampling was approved by the owner. CMT in the quarters enrolled in the study was >2 on a scale of 1-5 (Klastrup and Schmidt Madsen, 1974). Additional data comprised clinical signs, breed, date of birth, parity and the last date of calving. All samples were collected by the veterinarians and veterinary students of the Production Animal Hospital. The study had features of both cross-sectional and experimental study.

Data for study IV originated from an experimental challenge conducted at the Production Animal Hospital of the Faculty of the Veterinary Medicine (University of Helsinki, Mäntsälä) between January and May in 2008. Eight clinically healthy, primiparous and mid-lactating cows were infected with *Staphylococcus simulans* and *S. epidermidis* in a crossover study of four periods. One period included a two-week sampling period after the inoculation and a two-week wash-out period. Test quarters were sampled a week before inoculation in two consecutive samplings to ensure IMI-free status of the quarter with BC. No bacterial growth was detected in culture. SCC of the test quarters were <100 000 cells/ml and another healthy quarter of the cow served as a control. Experimentally infected quarters did not serve as test quarters again in following challenge periods. If quarters were positive in bacterial culture at the last sampling at 336h post challenge (PC), intramammary antibiotic were administered. *S. simulans* quarters were treated once a day for three days with procaine penicillin (Carepen®600mg, Vetcare Finland Oy). *S. epidermidis* quarters were treated accordingly with cloxacillin (Wedeclox mastitis® 1000mg, WDT, Germany). The treatments were chosen based on susceptibility testing. Treated quarters were tested and confirmed negative in bacterial culture in three consecutive samplings 11, 12 and 13 days post-treatment. All samples were collected, cultured and examined by authors Hiitiö, Simojoki and Taponen. Experimental infection (IV) was approved by the Ethics Committee of the University of Helsinki (HY 88-06). Preparation of the inoculates was described in Simojoki et al. (2011).

In the experimental studies, sample sizes were impacted by the availability of cows and budgetary limitations.

4.2. Cows and herds

More detailed information of the herds and cows of the studies is presented in Table 4. Background information in study I was not available for privacy and data protection reasons.

Table 4. Basic information for the sampled cows, herds they were in and sample numbers in studies I – IV.

Study	I	II	III	IV
Total number of samples	294	226	149	280
Number of sampled cows	n/a ¹	53	88	8
Number of sampled quarters	294	113	149	14
Number of herds	n/a	2	44	1
Size of the herds	n/a	420, 700	7-114	
Type of the barn	n/a	Tie-stall	Tie-stall, Free-stall	Tie-stall
Bedding material	n/a	Straw	Saw dust, Peat, Straw	Saw dust
Breed of the cows ²	n/a	100% Ho	27% Ho 63% NR 10% Other	87% Ho 13% NR
Average parity (median)	n/a	3	3	1
Average milk yield of the cow (kg)	n/a	27.6	n/a	3.8 ³

¹Information was not available

²Ho=Holstein, NR=Nordic Red, including registered as Ayrshire breed, Other= Mixed breeds, Finncattle and Jerseys

³Average milk yield of a quarter.

4.3. Milk sampling protocols

Two experimental sampling techniques were used to collect a quarter milk sample: sampling with a needle and vacuum tube (II) and sampling with a cannula (III), in addition to the conventional aseptic technique (I-IV). Sampling took place at random time points related to milking (I, III), prior to milking (IV) or within two hours of milking (II).

4.3.1. Aseptic milk sampling (I-IV)

Quarter milk samples were taken according to the internationally agreed guidelines (Middleton et al., 2017, Fig 1). Teats and udders were first cleaned with cotton towels if visibly dirty (II-IV) and foremilk was stripped. CMT was conducted for each test quarter before milk sampling (II-IV) and scored on a scale of 1-5 (Klastrup and Schmidt Madsen,

1974). Aseptic milk sampling (II-IV) was conducted according to the international guidelines (Hogan et al., 1999). The teat end was scrubbed with cotton moistened in 70% ethanol, at least three times or until visibly clean. A few strips of milk were discarded and the sample was milked into a 10 ml plastic milk tube without preservatives. The sampler wore disposable gloves (II-IV). This sampling protocol has been introduced and well adopted in Finland, yielding good quality samples (Vakkamaki et al., 2017), and we may assume that most quarters also in study I were sampled accordingly.



Fig 1. Aseptic milk sampling technique.

4.3.2. Needle sampling (II)

The sampling protocol was designed to obtain milk directly from the udder cistern (*Sinus lactifer, pars glandularis* (Schaller, 1992), to avoid possible contamination sources from the teat and related anatomical parts. Prior to sampling, the cows were sedated lightly with an intravenous dose of xylazine (0.02 mg/kg, Rompun 20 mg/ml, Bayer Animal Health GmbH, Germany) and 0.5 ml of oxytocin (Vetox 10 IU/ml, Vetcare Oy, Finland) was simultaneously administered intravenously to induce milk ejection. The site of puncture was randomly chosen by the sampler, targeting the easiest puncture line possible and avoiding visible veins (Fig 2). An area of about 3 x 3 cm at the puncture site was scrubbed with cotton, moistened in 70% ethanol, at least three times or until visibly clean. After cleaning the site, the sampler wore a new pair of plastic disposable gloves. A 20-Gauge double-ended needle (Venoject Multi-Sample, Terumo Europe N.V., Leuven, Belgium) was inserted from

the puncture aimed towards the udder cistern. After placing the needle, a 9 ml vacuum tube without preservatives (Vacuette Tube Z, Greiner Bio-One, Monroe, NC) was attached and the milk sample was drawn into the tube. The needle was removed and the site pinched or pressured for a while if bleeding was apparent. Needle samples were taken immediately after conventional sampling.



Fig 2. Needle sampling technique.

4.3.3. Cannula sampling (III)

The aim of the technique was to bypass the teat orifice and teat canal to collect the milk from the teat cistern (*Sinus lactifer, pars papillaris*; Schaller, 1992). In addition to disinfecting the teat for conventional sampling, the teat end was scrubbed again in a similar manner with 70% ethanol before inserting the teat cannula (Fig 3). Cannulas were individually packed, sterile teat cannulas 42 mm in length (Teat cannula, Vetcare Finland Oy). Milk was allowed to flow freely into a plastic milk tube without preservatives (Linkoputki 16 x 100mm Plastone, Mekalasi Oy, Finland). The sampler wore a new pair of disposable gloves before inserting the cannula. The cannula samples were taken after conventional samples.



Fig 3. Cannula sampling technique.

4.4. Inoculation and test protocol in experimental challenge (IV)

S. simulans and *S. epidermidis* strains of the inoculate originated from natural persistent infections. The strains were identified with API Staph ID 32 (bioMérieux, Marcy l’Etoile, France) and amplified fragment length polymorphism (AFLP). One challenge dose contained 5.7×10^6 cfu in 7 ml of saline. The inoculation was done on day zero, thirty minutes after the morning milking. At every sampling, a few strips of foremilk were discarded first, followed by visual inspection of the milk on a CMT plate and CMT testing. Aseptic milk samples for bacteriological culture, PCR testing and SCC determination were collected at 0, 4, 6, 12, 21, 27, 30, 36, 45, 54, 69, 78, 93, 102, 117, 126, 141, 168 (7 d), 240 (10 d) and 336 hours (14 d) post-challenge (PC). Sampling was carried out always prior to milking.

4.5. Transportation and handling of milk samples (I-IV)

Samples in study I were sent to the regional laboratory of Valio Ltd. (Lapinlahti, Finland), cooled below 6 °C within 48 h from sampling, whereupon they were immediately

transported refrigerated to the national mastitis reference laboratory, Finnish Food Safety Authority Evira (Kuopio, Finland). Samples were cultured on arrival. Two aliquots were separated and frozen to -20 °C immediately after bacterial culture.

In Estonia (II), sampling took place before noon. Samples were cooled on ice and transported in cooler boxes to the laboratory of the Department of Production Animal Medicine (Faculty of Veterinary Medicine, University of Helsinki, Finland) within 8 hours from sampling and stored in a refrigerator at 6 °C. The following morning, samples were cultured. In addition, two 2.5 ml milk aliquots were drawn aseptically from all samples (Vacuette Tube Z, 4 ml) and stored at -20 °C for PCR and milk N-acetyl- β -D-glucosaminidase (NAGase) activity determination.

Samples for study III were transported to the laboratory (Department of Production Animal Medicine, University of Helsinki) within four hours of sampling. Samples were cultured to obtain a BC result for the client. Those results were not included in study III. For PCR, carried out later, 2.5 ml of milk was separated and stored at -20 °C in milk tubes (Vacuette Tube Z, 4 ml, Greiner Bio-One).

Milk samples from experimental infections (IV) were cultured after sampling and aliquots were frozen at -80 °C for PCR and other purposes.

No preservatives were included in any of the milk samples (I-IV).

4.6. Bacterial culture (I, IV)

Milk samples were cultured in two studies included in this thesis, studies I and IV. Culturing in study I was carried out at the Finnish Food Safety Authority Evira (Kuopio, Finland) and samples of the experimental infection (IV) were cultured in the laboratory of the Department of Production Animal Medicine (Faculty of Veterinary Medicine, University of Helsinki, Finland).

Bacterial cultures in Evira (I) were carried out in an in-depth manner, where all colony forming units (cfu) were enumerated and identified if possible, also from samples containing >2 species, which would normally be defined as contaminated (Middleton et al., 2017). Morphologically different colonies were counted and identified with suggested protocols (Middleton et al., 2017). Gram staining, catalase test, oxidase test (Pro-Lab Diagnostics, Richmond Hill, ON, Canada), API Staph, API ID32 Strep, API 20 E and API Coryne (bioMérieux, Marcy l'Etoile, France) were used in the identification schemes. For *Staphylococcus* spp., a positive coagulase test (BBLTM Rabbit plasma, BD Diagnostics, USA) was confirmed with API Staph (bioMérieux, Marcy l'Etoile, France). Eosin methylene blue agar (EMB) (Oxoid, Basingstoke, Hampshire, UK) was used for detection of *E. coli* and TSA was supplemented with Tween-80 (0.1%) (Amresco, Solon, OH, US) for detection of lipophilic *Corynebacterium bovis*.

The volume of cultured milk was 0.01 ml in study I and 0.1 ml in study IV. In both studies, milk was spread using a conventional loop over the entire 9 cm plate and incubated at 37°C for 48 hours. Additionally, in study IV, several dilutions of milk were cultured to ensure correct colony counts in samples suspected of having large numbers of colonies. Plates were inspected at 24 and 48 hours. If colony count exceeded 100 on a plate, those colonies were recorded as 100 in study I. In experimental study IV number of cfu was counted using dilution series. Consequently, the detection limit was 100 cfu/ml (I) or 10 cfu/ml (IV). An additional higher detection threshold (500 cfu/ml) for a positive result was used for coagulase negative staphylococci (I).

In study IV, especially on plates where growth was dense, the morphology or the size of the colonies can vary. Morphologically different colonies on sample plates observed during the study were identified as coagulase negative staphylococci with standard identification schemes mentioned above and checked with API Staph ID 32 test (bioMérieux) (Simojoki et al., 2011). The API Staph profiles were compared with the API Staph profiles from the original AFLP confirmed *S. epidermidis* and *S. simulans* strains used in challenge.

4.7. Indicators for inflammation

Somatic cell count (SCC, II-IV) and N-acetyl-β-D-glucosaminidase (NAGase, I) activity of the quarter milk were used as indicators of inflammation. Cow composite milk SCC from DHI samples were also used as selection criteria in enrolment of the cows (II).

4.7.1. Somatic cell count, SCC

SCC were evaluated with CMT (II-IV) on a scale of 1-5 (Klastrup and Schmidt Madsen, 1974) on site. More precise results for SCC were determined using the optical DeLaval cell counter DCC (II, III) (DeLaval Inc., Tumba, Sweden) or a fluoro-optical method using the Fossomatic instrument (Valio Ltd Laboratories, Finland) (IV). The upper quantification limit of Fossomatic SCC analysis was 10×10^6 cells/ml.

4.7.2. N-acetyl-β-D-glucosaminidase activity in the milk

NAGase activity of the milk samples was determined in the laboratory of the Department of Production Animal Medicine (University of Helsinki, Mäntsälä, Finland) using an in-house microplate modification (Hovinen et al., 2016) of the fluorogenic method (Mattila and Sandholm, 1985). The results were expressed as picomoles of 4-MU/min/μl milk at 25 °C. The maximum detection limit of NAGase activity is 24.49 pmol 4-MU/min/μl milk. The mean NAGase activity for healthy quarters (SCC < 100 000 cells/ml) has been reported as

0.45 pmol 4-MU/min/ μ l milk (range 0.09–1.04 pmol 4-MU/min/ μ l milk) (Hovinen et al., 2016). The definition of mastitic milk in study I was the result exceeding the upper range value for normal milk (>1.04 pmol 4-MU/min/ μ l milk).

4.8. Multiplex real-time PCR (I-IV)

All samples for PCR were examined at the laboratory of Thermo Fisher Scientific Ltd. (Vantaa, Finland) by the manufacturer of the PathoProofTM Mastitis PCR assay (I-IV). Samples were delivered to the laboratory deep frozen and thawed before the PCR analysis. Two different kits, PathoProofTM Mastitis Complete-12 Kit (I, IV) and PathoProofTM Mastitis Complete-16 Kit (II, III), were used. Kit components and PCR machinery compatible with PathoProofTM Mastitis was used according to the instruction manuals (Thermo Fisher Scientific). All the samples were delivered to the PCR laboratory labelled only by ID number of the sample. No information on the origin, background or sampling technique was given to the laboratory.

4.8.1. Target pathogens

Oligos of the Mastitis Complete-12 Kit are designed to target DNA of the following species or group of species: *C. bovis*, *Enterococcus* spp. including *Enterococcus faecalis* and *E. faecium*, *E. coli*, *Klebsiella* spp. including *K. oxytoca* and *K. pneumoniae*, *S. marcescens*, *S. aureus*, *Staphylococcus* spp. (non-aureus staphylococci), *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis*, *T. pyogenes* and/or *P. indolicus*. PathoProofTM Mastitis Complete-16 Kit includes additional oligos for *M. bovis*, *Mycoplasma* spp., *Prototheca* spp., and yeasts. In addition, both test kits have oligos for staphylococcal beta-lactamase gene (*blaZ*).

Enterococcus spp., *Klebsiella* spp., *Mycoplasma* spp., *Prototheca* spp. and *Staphylococcus* spp. were identified to group level. PathoProofTM Mastitis Complete Kits have joined oligos for *Mycoplasma* spp. and for *Staphylococcus* spp. In addition, specific oligos for *S. aureus* and *Mycoplasma bovis* are included. To differentiate *Staphylococcus* spp. from *S. aureus* positive sample, or a *Mycoplasma* spp. from a *M. bovis* positive sample, a 3-cycle difference in their Ct values is required. PathoProofTM Mastitis PCR Assay reports non-aureus staphylococci (NAS) as *Staphylococcus* spp. Thus, *Staphylococcus* spp. results from PCR throughout this thesis are considered as non-aureus staphylococci without exceptions.

4.8.2. Sample volume and reactions

The sample volume used in PCR was 350 μ l, which was concentrated to 100 μ l in the elution step of the extraction protocol (I-IV). A total of 17.5 μ l (I) or 20 μ l (II-IV) of

extracted DNA was used in PCR. In both PathoProof^f™ Mastitis Complete Kits microbial targets are divided into four reactions, leading to a volume of 5 µl of extracted DNA solution in every reaction. Each reaction detects three (Complete-12) to four targets (Complete-16) and an internal amplification control (IAC). In the Complete-12 Kit, targets are divided into four reactions as follows: *S. aureus*, *Enterococcus* spp., *C. bovis* and an IAC (reaction 1), *blaZ*, *E. coli*, *S. dysgalactiae*, and an IAC (reaction 2), *Staphylococcus* spp., *S. agalactiae*, *S. uberis* and an IAC (reaction 3) and *Klebsiella* spp., *Serratia marcescens*, *T. pyogenes/P. indolicus* and an IAC (reaction 4).

In PathoProof^f™ Complete-16 Kit the first reaction primer mix includes oligos for *S. aureus*, *Enterococcus* spp., *C. bovis*, *M. bovis* and an IAC, second reaction oligonucleotides for *blaZ*, *E. coli*, *S. dysgalactiae*, *Mycoplasma* spp. and an IAC. Reaction three contains oligos for *Staphylococcus* spp., *S. agalactiae*, *S. uberis*, *Prototheca* spp. and an IAC and fourth reaction oligos for *Klebsiella* spp., *Serratia marcescens*, *T. pyogenes/P. indolicus*, yeast and an IAC.

IAC template DNA was included in every reaction. According to the manufacturer, if IAC in reaction is not in the acceptable range, an error in the process is suspected and the PCR is run again or the sample is prepared from the start. In addition, if negative controls yield a positive result, laboratory contamination should be suspected and the sample tested again. Every PCR run included at least one negative control (Thermo Fisher Scientific Ltd., 2017). Samples should be retested also if fluorescence curves are not optimal, indicating a possible error in the process. Study samples were assessed according to these guidelines (personal communication 2017, Mika Silvennoinen Thermo Fisher Scientific Ltd.).

4.8.3. Cycle threshold values

Maximum Ct value, i.e. a maximum of cycles in PCR test, was 40.0 (I-IV) for each species or group and the *blaZ* gene reported to an accuracy of one decimal place. A definition for a positive result in routine use of the PCR assay is a Ct value of ≤37.0 (I, III). In addition to Ct 37.0, two other cut-offs of 34.0 (II, IV) and 40.0 (II) and their effects on results were tested.

The tested cut-off values of 34.0, 37.0, and 40.0 were chosen because Ct 40.0 represents the maximum detection limit and Ct 37.0 is the routine cut-off limit in the PathoProof^f™ Mastitis PCR Assay. A Ct limit of 34.0 should show a similar decline in the amount of amplified DNA from Ct 37.0 as the decline from Ct 40.0 to 37.0. An approximately 3.3-cycle difference in Ct values translates to a ~10-fold difference in the concentration of the target DNA.

In PathoProof^f™ Mastitis software, Ct ranges for low, intermediate and high amount of detected DNA have been created for each target pathogen. These interpretation ranges are modified according to manufacturer's own unpublished data.

4.8.4. Genome copy numbers

Ct values were converted into genome copy (gc) numbers for comparison with bacterial culture results (cfu/ml) (I, IV). Gc/reaction were provided by Thermo Fisher Scientific according to the authors' request. Ct values were transformed into genome copy numbers using the following formula: genomic copy number per reaction = $10^{(Ct-x)/y}$, where Ct = cycle threshold value, x = slope of a standard curve and y = intercept of a standard curve. The formula does not take into account possible differences in target gene or DNA fragment copy numbers within a genome.

4.9. Data analyses

4.9.1. Sensitivities and specificities (I)

Relative specificity (Sp) and sensitivity (Se) for PCR were calculated using bacterial culture as the reference (gold standard) in diagnosing *S. aureus* and *Staphylococcus* spp. in study I. For the calculation, BC results and PCR results were converted into binary outcomes, positive intramammary infection (IMI) yes (1) or no (0). Only samples including one or two species were included and contaminated culture samples and the respective PCR samples were excluded from these calculations (n=256, I). Definition of a positive result for *S. aureus* was 100 cfu/ml and for *Staphylococcus* spp. 500 cfu/ml, based on the national recommendations (Honkanen-Buzalski and Seuna, 1995).

PCR results were considered IMI positive (1) when the Ct value of the targeted pathogen was ≤ 37.0 and negative (0) when Ct value exceeded 37.0 (I). If the sample included both *Staphylococcus* spp. and *S. aureus*, according to previous definitions, the sample was recorded as *S. aureus* positive and *Staphylococcus* spp. positive (I).

Predictive values, taking into account also the prevalence of the mastitis among the data, were calculated in study I for *S. aureus* and for *Staphylococcus* spp. Positive predictive value (PPV) results in probability of the cow with a positive test result actually having an IMI. Negative predictive value (NPV) results in the probability of cow with a negative test result truly being free from IMI.

4.9.2. Agreement between the diagnostic tests

Agreement between BC and PCR in providing a positive IMI diagnosis was tested with Cohen's Kappa (κ)-coefficient (I, IV). For these calculations, the BC and PCR results were converted into binary outcomes of positive IMI, yes (1) or no (0). κ -coefficient was also used in testing agreement between the two sampling techniques in detecting most common microbes (III) and separately in detecting *C. bovis* and *Staphylococcus* spp. (II). Values 0–

0.20 were characterized as slight, 0.21–0.40, fair, 0.41–0.60, moderate, 0.61–0.80, substantial, and in almost perfect agreement, 0.81–1 (McHugh, 2012).

The effect of the sampling technique (conventional vs. cannula) on the proportion of different species found in the milk samples was tested with a Pearson's chi square test (III). The test was run only for those species for which there was at least one detection and with 80% of 2 x 2 tables including over 5 detections (Dohoo et al., 2010).

4.9.3. Comparison of the mean amounts of detected target pathogen DNA (II, III)

The differences in the amount of detected target pathogen DNA by conventional and needle sampling techniques were tested separately for the most frequently detected species, *C. bovis* and *Staphylococcus* spp. (II). Samples were considered paired because the origin of the sample was the same quarter and only the sampling technique changed (II, III). Differences in means of recorded Ct values were calculated for the data separately for all three Ct cut-off values 34.0, 37.0 and 40.0 (II) using t-tests for paired samples. The results were significant if the *p*-value was <0.05.

In study III, Student's t-test for paired samples was used to compare the mean amounts of detected target pathogen DNA. Only samples containing less than three different species per sample were included in statistical calculations (III). The results were considered significant if the *p*-value was <0.05.

4.9.4. Correlations (I, II, IV)

Correlations between cfu/ml and genome copy number (gc)/ml were tested with Spearman's rank correlation coefficient because the data were not normally distributed (I, IV). In study I, correlations between cfu/ml and gc/ml were calculated for *Staphylococcus* spp. and for *S. aureus*. Only samples which were positive with both methods, PCR and BC, were included (n=70 for *S. aureus* and n=75 for *Staphylococcus* spp.). The results were considered significant at *p* < 0.05.

In study IV, correlations were calculated for the whole two-week period (0 – 336 h), but also for the acute phase of 0 – 36 h, the subsequent phase up to one week (45 – 168 h) and the second week, including sampling at 240 and 336 h PC. Correlations were calculated for samples originating from quarters infected with *Staphylococcus* spp. (i.e., both species together, n=280) and for *S. simulans* (n=120) and *S. epidermidis* (n=160) separately. Correlations between cfu/ml and gc/ml for both species were also shown separately for each individual cow. Due to the variation among cows, low statistical power due to small sample size and correlated data structure, *p*-values were left unreported (IV).

In study II, Spearman correlation was used to compare Ct values in paired samples taken with conventional aseptic sampling technique and with experimental needle technique. Correlations were calculated for the data with three different Ct cut-off values of 34.0, 37.0 and 40.0.

4.9.5. Statistical significance and used programs

In all statistical analyses, $p < 0.05$ was considered to represent the level of statistical significance (I-IV). Analyses were conducted with IBM SPSS Statistics for Windows, Version 20.0. (I), Version 21.0. (II) Armonk, NY: IBM Corp. and IBM SPSS Statistics Version 24.0. for Macintosh (IV) Armonk, NY: IBM Corp. Stata Version 11 (Stata Statistical Software: Release 11. College Station, TX: StataCorp LP.) was used in study III.

4.9.6. Nomenclature

Mastitis is defined as an inflammation of the parenchyma of the mammary gland (Zhao and Lacasse, 2008) and IMI as presence of pathogenic micro-organisms in the secretory tissue, the duct and tubules of the bovine mammary gland (International Dairy Federation, 2011). Our definition throughout the thesis is that mastitis includes elevated indicator levels of inflammation and is caused by IMI. In study I, where the SCC status was not available, we assume that farmers sent samples only when SCC was high or there were some other clear indicators leading to suspected mastitis. In study II, CMT was performed for all tested quarters. Intentionally, fourteen quarters with CMT of one were included. In study III, all the cases were classified as mastitis where veterinary consultancy was needed. In study IV, we induced mastitis in every quarter.

A result from multiplex real-time PCR assay is a Ct value. This indicates the number of cycles needed to detect the fluorescence of a target pathogen DNA. The Ct cut-off may be adjusted according to purpose. A PCR result was considered positive (PCR+) when target DNA was amplified from the sample with a Ct value under the set Ct cut-off value. PCR negative (PCR-) samples were defined as samples with no detected target pathogen DNA within the limits of the set cut-off value. The Ct cut-off for a positive PCR result and the spectrum of the used panel has been reported in each study separately.

No agreed standard for contaminated sample is available in PCR. If the sample contained DNA of more than two target pathogens, we used the term ‘multispecies sample’.

5 Results

The results section comprises four parts. The first part is focused on comparison of culture and PCR results, including elimination of bacteria from infected quarters. The second part includes the effect of different sampling methods on PCR results. The third part presents the effect of Ct cut-off value on the results with respect to detected target pathogens, as well as the mean pathogen-specific Ct values. Results regarding inflammation indicators are presented in the last section.

5.1. Comparison of multiplex real-time PCR and conventional culture (I, IV)

The proportion of samples positive for DNA of the target species (83%) in PCR was lower than proportion of samples positive in culture (85.3% 251/294) in natural IMI, in study I (Table 5). Most of the samples included only one or two different species (Table 5). No target DNA was detected in 17% of samples examined using PCR and 15% of the cultured samples yielded a no-growth result (Table 5).

Table 5. Number and proportions of samples with different number of species detected with conventional culture (BC) or multiplex real-time PCR (PCR) from study I. Definition for a positive result in PCR was a sample with target DNA detected with a Ct value of ≤ 37.0 (PathoProof™ Complete-12 Kit). Definition of a positive BC result was ≥ 100 cfu/ml. Samples were aseptic quarter milk samples originating from natural IMI, collected mainly by farmers.

Species detected per sample	Study I			
	BC		PCR	
	n	%	n	%
0	43	14.6	50	17.0
1	161	54.8	161	54.8
2	52	17.7	59	20.1
3*	30	10.2	18	6.1
4*	6	2.0	5	1.7
5*	2	0.7	1	0.3
Total	294	100.0	294	100.0

*Considered contaminated in the conventional bacterial culture

In PCR results, the most prevalent species detected were *Staphylococcus* spp. (NAS) (n=113), *S. aureus* (n=79), *Str. dysgalactiae* (n=39) and *Str. uberis* (n=38) (Table 6). *Staphylococcus* spp. (NAS) were present as the only detected pathogen in 53% (60/113) and with one other pathogen in 32% of the samples. The second most prevalent pathogen, *S. aureus*, was present in 66% (52/79) as a single detection and in 24% (19/79) with one other species (Table 6).

Table 6. Total number of target pathogens from multiplex real-time PCR (I). The first column shows the total number of samples where the species was detected. The subsequent columns (1 - 5) indicate the total number of different species in the sample.

	Study I					
	Total number of species detected by PCR					
	n _{total}	1	2	3	4	5
<i>Corynebacterium bovis</i>	28	4	17	5	2	0
<i>Enterococcus</i> spp.	7	2	1	2	1	1
<i>Escherichia coli</i>	17	7	6	3	1	0
<i>Klebsiella</i> spp.	6	1	3	1	1	0
<i>Serratia marcescens</i>	1	1	0	0	0	0
<i>Staphylococcus aureus</i>	79	52	19	6	1	1
<i>Staphylococcus</i> spp. (NAS)	113	60	36	12	5	0
<i>Streptococcus agalactiae</i>	1	0	1	0	0	0
<i>Streptococcus dysgalactiae</i>	39	14	13	9	2	1
<i>Streptococcus uberis</i>	38	13	11	9	4	1
<i>Trueperella pyogenes/Peptoniphilus indolicus</i>	29	7	11	7	3	1

The most prevalent finding in BC results was coagulase negative staphylococci (CNS), followed by *S. aureus* (Table 7). Among all CNS positive samples (n=103), CNS was the only species found in 49.5% (51/103) and accompanied a high number of samples containing two or more pathogens (Table 7). In contrast, in 67.1% (49/73) of *S. aureus* positive samples, *S. aureus* was the only species found (Table 7).

The proportion of BC samples with more than two species, which are considered contaminated according to the NMC interpretation guidelines (Middleton et al., 2017), was 13% (38/294) with a detection limit of 100 cfu/ml (I) (Table 5). In PCR results, the corresponding figure was 8% (24/294), examined with a target panel of 11 pathogens and a Ct cut-off 37.0 (Table 5). *Staphylococcus* spp. were most commonly found in contaminated BC samples (61%, 23/38), followed by *Corynebacterium* spp., *Aerococcus* spp., *Bacillus* spp. and *Str. dysgalactiae*. The most prevalent pathogens in the multispecies samples tested with PCR were also *Staphylococcus* spp., followed by *Str. uberis* and *T. pyogenes*.

Corynebacterium spp. other than *C. bovis*, *Aerococcus* spp. and *Bacillus* spp. represented the most frequent findings not included to the PCR test panel (Table 7). These pathogens were mostly cultured from samples defined as being contaminated in BC.

Table 7. The culture results from 294 samples of (I). Definition of a positive culture was 100 cfu/ml. The first column shows the total number of samples where the species was detected. The subsequent columns (1 - 5) indicate the total number of different species in the sample. For example, *Aerococcus* spp. were detected in 20 samples and 14 of those samples were contaminated. Species not detectable with PCR, e.g. not included in the PCR test panel (PathoProof™ Complete-12 Kit), are highlighted in light grey.

	n _{total}	Number of different species in a sample, study I				
		1	2	3*	4*	5*
<i>Aerococcus</i> spp.	20	3	3	11	3	0
<i>Bacillus</i> spp.	12	2	1	7	2	0
<i>Chryseobacter</i> spp.	3	0	0	0	2	1
<i>Coagulase negative staphylococci</i> ²	103	51	29	16	5	2
<i>Corynebacterium</i> spp.	35	1	15	14	3	2
<i>Corynebacterium bovis</i>	21	10	8	3	0	0
<i>Corynebacterium ulcerans</i>	1	1	0	0	0	0
<i>Enterobacter</i> spp.	1	0	0	1	0	0
<i>Enterococcus casseliflavus</i>	1	1	0	0	0	0
<i>Enterococcus</i> spp.	10	3	1	4	1	1
<i>Escherichia coli</i>	11	7	2	1	1	0
<i>Gemella</i> spp.?	1	0	0	0	1	0
<i>Globicatella</i> spp.	2	0	0	2	0	0
<i>Klebsiella</i> spp.	6	4	1	1	0	0
<i>Lactobacillus</i> spp.	1	0	0	1	0	0
<i>Lactococcus</i> spp.	6	1	3	0	1	1
<i>Leuconostoc</i> spp.?	1	0	0	1	0	0
<i>Micrococcus</i> spp.	1	0	0	1	0	0
<i>Moraxella</i> spp.	1	0	1	0	0	0
<i>Raoultella</i> spp.?	1	0	0	0	1	0
<i>Serratia marcescens</i>	1	0	1	0	0	0
<i>Staphylococcus aureus</i>	73	49	17	6	1	0
<i>Streptococcus agalactiae</i>	1	1	0	0	0	0
<i>Streptococcus bovis</i>	5	0	4	1	0	0
<i>Streptococcus dysgalactiae</i>	27	11	7	7	2	0
<i>Streptococcus uberis</i>	19	11	5	1	2	0
<i>Trueperella pyogenes</i>	16	5	5	4	2	0
Unidentified Gram-positive cocci	4	0	2	1	1	0
Unidentified rod	2	0	0	2	0	0
Yeast ³	4	2	1	1	0	0

n_{total} Total number of findings of the species/group of species

² In PCR these are referred as *Staphylococcus* spp. i.e. non-aureus staphylococci

³ Included in current PathoProof™ Complete-16 Kit

* Contaminated sample (>2 different species)

Table 8. Cross-tabulation of results positive or negative in PCR and bacterial culture in study I. Ct cut-off value for a positive result was 37.0 (PathoProof™ Complete-12 Kit) and the detection limit for a bacterial culture was ≥ 100 cfu/ml.

	PCR+	PCR-	Total
BC+	224	27	251
BC-	20	23	43
Total	244	50	294

About half (47%, 20/43) of the culture negative samples of study I were positive for PCR (Table 8), *Staphylococcus* spp. (NAS) representing the most prevalent species (Fig 4). Most of these PCR+/BC- results were detected in PCR with Ct values >32.0 (Fig 4), which indicate low bacterial counts according to the manufacturer. *Str. dysgalactiae* was an exception (Fig 4).

Over 50% of the PCR negative samples showed microbial growth in culture (Table 8). One out of these 27 PCR-/BC+ samples was considered contaminated. In 48% (13/27) the cultured species was not included in the target panel of the PCR assay. The number of cfu/ml in PCR-negative samples varied between 100 and $\geq 10\ 000$ cfu/ml (mean 1 281 cfu/ml, median 200 cfu/ml). *Staphylococcus* spp. were cultured most often from PCR- samples (30%, 15/50), with bacterial counts varying from 100 to 500 cfu/ml (Fig 5).

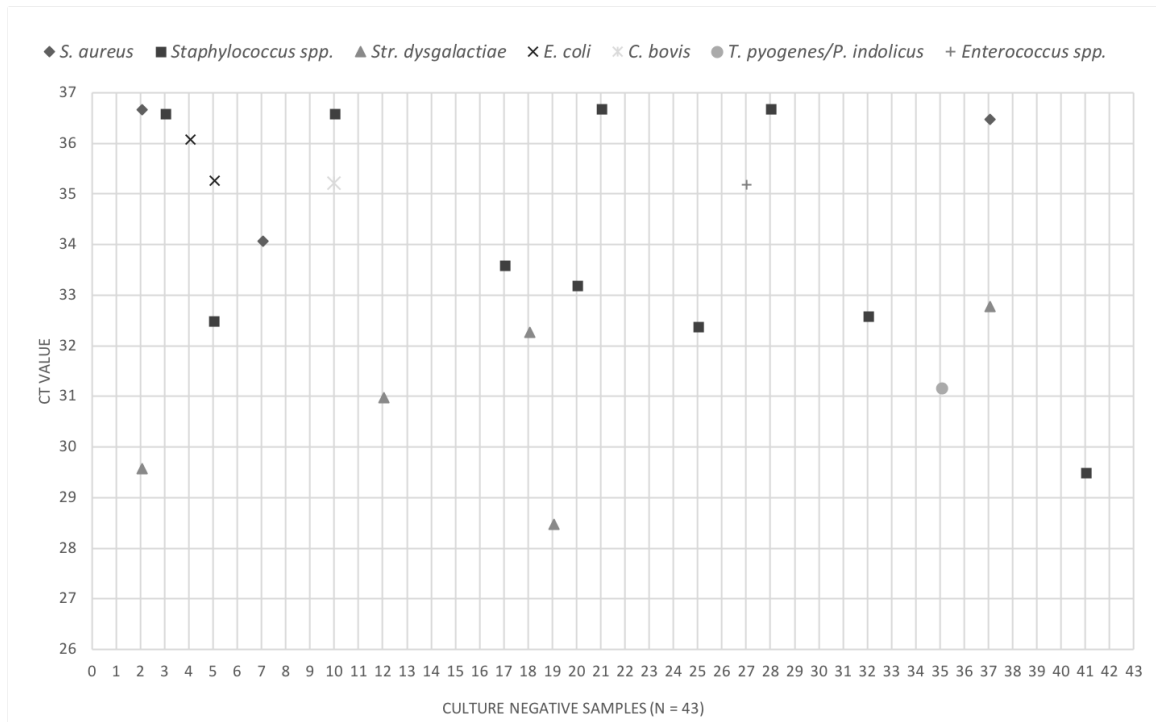


Fig 4. Species detected with PCR (n=20) from milk samples with no growth in the conventional bacteriological culture (43/294) (I). Ct cut-off for a positive PCR detection was 37.0. Each vertical line represents a single culture negative sample.

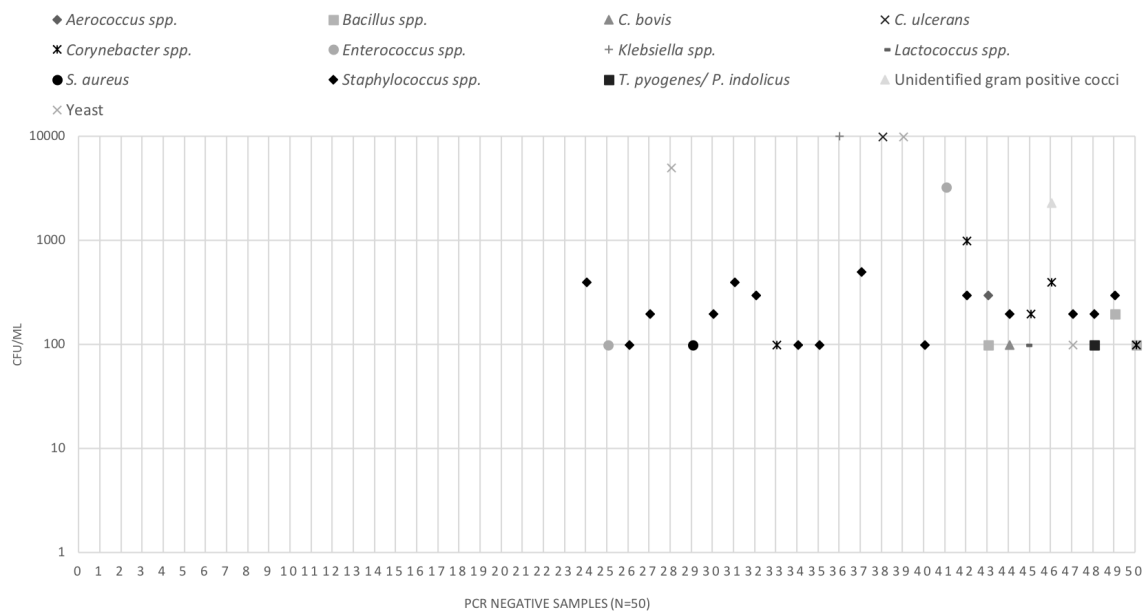


Fig 5. Cultured species and their bacterial counts (n=27) for quarter milk samples negative for any target-DNA in multiplex real-time PCR assay (50/294), study I. Detection limit for a positive culture result was ≥ 100 cfu/ml. Each vertical line represents a single sample.

5.1.1. Comparison of the BC and PCR results in experimental CNS challenge

Only two different coagulase negative staphylococcal species were used in the experimental challenge (IV). The PCR panel was the same as in study I, which does not differentiate these two species, but presents the result as *Staphylococcus* spp. A total of 280 samples were examined, 160 originating from quarters infected with *S. epidermidis* and 120 from quarters infected with *S. simulans*. In BC, a total of 80% (224/280) were positive for CNS and 90% (253/280) of the samples positive for *Staphylococcus* spp. in PCR (Ct cut-off 37.0) (Table 9). Of the *S. epidermidis* infected quarters, 75% (120/160) were positive in BC and 88% (140/160) in PCR (Ct cut-off 37.0). For *S. simulans* the corresponding figures were 87% (104/120) and 93% (112/120) (Table 9).

Table 9. Number of detections in multiplex real-time PCR (PCR) and bacterial culture (BC) from the *Staphylococcus* spp. experimentally infected quarters. The results are presented with two different Ct cut-off values, 37.0 and 34.0. Agreement between PCR and BC is reported with Cohen's Kappa. All results are presented for the whole two-week study period (0 - 336 h) and separately for the first 36 h, the subsequent 45 - 168 h and 240 - 336 h post challenge (PC). All Cohen's Kappa results were statistically significant ($p < 0.05$), except for two results marked in the table.

Hours	PC ¹	Species	Ct 37.0				Cohen's Kappa	Ct 34.0				Cohen's Kappa
			BC+	BC-	PCR+	PCR-		BC+	BC-	PCR+	PCR-	
			PCR+	PCR-	PCR-	PCR+		PCR+	PCR-	PCR-	PCR+	
0 - 336		<i>Staphylococcus</i> spp.	218	6	21	35	0.42	200	24	32	24	0.46
		<i>S. epidermidis</i>	114	6	14	26	0.36	101	19	24	16	0.43
		<i>S. simulans</i>	103	1	7	9	0.54	100	4	8	8	0.49
0 - 36		<i>Staphylococcus</i> spp.	94	2	14	2	0.85	91	5	14	2	0.76
		<i>S. epidermidis</i>	55	1	8	0	0.93	53	3	8	0	0.82
		<i>S. simulans</i>	39	1	6	2	0.76	38	2	6	2	0.70
45 - 168		<i>Staphylococcus</i> spp.	103	4	3	30	0.07 ²	91	16	14	19	0.28
		<i>S. epidermidis</i>	55	4	3	18	0.09 ²	46	13	10	11	0.25
		<i>S. simulans</i>	48	0	0	12	-	45	3	4	8	0.32
240 - 336		<i>Staphylococcus</i> spp.	20	1	4	3	0.58	18	3	4	3	0.43
		<i>S. epidermidis</i>	10	1	3	2	0.54	8	3	3	2	0.31 ²
		<i>S. simulans</i>	10	0	1	1	0.63	10	0	1	1	0.63

¹Post challenge

²Not statistically significant, $p > 0.05$

Of the 56/280 culture negative samples in study IV, 63% (35/56, Ct 37.0) and 43% (24/56, Ct 34.0) were positive in PCR for *Staphylococcus* spp. (Table 9). In results originating from quarters infected with *S. epidermidis*, a total of 14% (40/280) samples were negative in BC. Of these BC negative *S. epidermidis* samples, PCR detected DNA of *Staphylococcus* spp. from 65% (26/40, Ct 37.0) and 40% (16/40, Ct 34.0). From BC negative *S. simulans* samples (13%, 16/120), 56% (9/16, Ct 37.0) and 50% (8/16, Ct 34.0) gave a positive *Staphylococcus* spp. detection in PCR (Table 9). The majority of these BC-/PCR+ detections occurred between 50 – 150 h post challenge with recorded Ct values of >30.0. SCC of those quarters varied between 5 000 and 10×10^6 cells/ml (mean 2.0×10^6 , median 519 000 cells/ml of milk) (IV).

Only 3% (6/224) of the samples where the staphylococcus strains were detected in BC were PCR- negative for *Staphylococcus* spp. with a Ct cut-off of 37.0 Bacterial counts (BC) in these six samples varied from 20 to 1 090 cfu/ml (median 95 cfu/ml). With the lower Ct cut-off value of 34.0, 10% (23/224) of the culture positive samples were negative in PCR, with bacterial counts in BC ranging between 10 and 1 870 cfu/ml (median 140 cfu/ml).

5.1.2. Sensitivity (Se), specificity (Sp) and predictive values (I)

Relative Se and Sp of the PCR assay for detecting *S. aureus* or *Staphylococcus* spp. were calculated assessing the culture as the reference (gold standard). Se and Sp for *S. aureus* was 97.0% and 95.8%. Se and Sp for PCR detecting *Staphylococcus* spp. was 86.7% and 75.4%. Positive predictive value for *S. aureus* was 88.9% and negative predictive value 98.9%. For *Staphylococcus* spp. the corresponding figures were 42.9% and 96.4%.

5.1.3. Agreement between BC and PCR in detecting pathogens (I, IV)

In study I with natural IMI (n=294) and with a BC detection limit of 100 cfu/ml, *S. aureus* was cultured only once from a PCR negative sample (Ct cut-off 37.0) and detected with PCR from three culture negative samples. The agreement (κ -coefficient) between the two diagnostic tests in detecting *S. aureus* was 0.90 ($p < 0.05$), indicating almost perfect agreement (I). With *Staphylococcus* spp. (NAS) in natural IMI and BC detection limit of 500 cfu/ml, the agreement between the tests was 0.44 ($p < 0.05$), considered moderate agreement (I).

In the experimental challenge study IV, the agreement between the tests detecting *Staphylococcus* spp. was almost perfect ($\kappa = 0.85$) for the first 36 h after the challenge when PCR results were assessed with Ct cut-off 37.0 and substantial ($\kappa = 0.76$) with cut-off 34.0 (IV). For the whole 336 h study period, a moderate agreement was achieved (BC detection limit 10 cfu/ml) (IV). Agreement between BC and PCR for the whole 336 h was better for *S. simulans* than for *S. epidermidis* and the two tests agreed better with Ct cut-off 37.0 than with 34.0.

5.1.4. Correlation between genome copy numbers from PCR and colony forming units from BC (I, IV)

Correlation between gc/ml and cfu/ml for *S. aureus* was 0.68 ($p < 0.05$, I). From fourteen samples with PCR indicating low amount ($Ct > 30.1$) of *S. aureus* DNA according to manufacturers' interpretation guideline, over 2 000 cfu/ml were cultured. In five of those samples the *S. aureus* counts exceeded 10 000 cfu/ml (I). Correlation between gc/ml and cfu/ml in natural *Staphylococcus* spp. IMI was only moderate to poor (0.47, $p < 0.05$, I). Figures 6 and 7 show all the results for *S. aureus* (n=81) (Fig 6.) and *Staphylococcus* spp. (Fig 7) which were positive with BC, PCR or with both. To see the scatterplot based on the correlation values, please see the Fig 4 in study I.

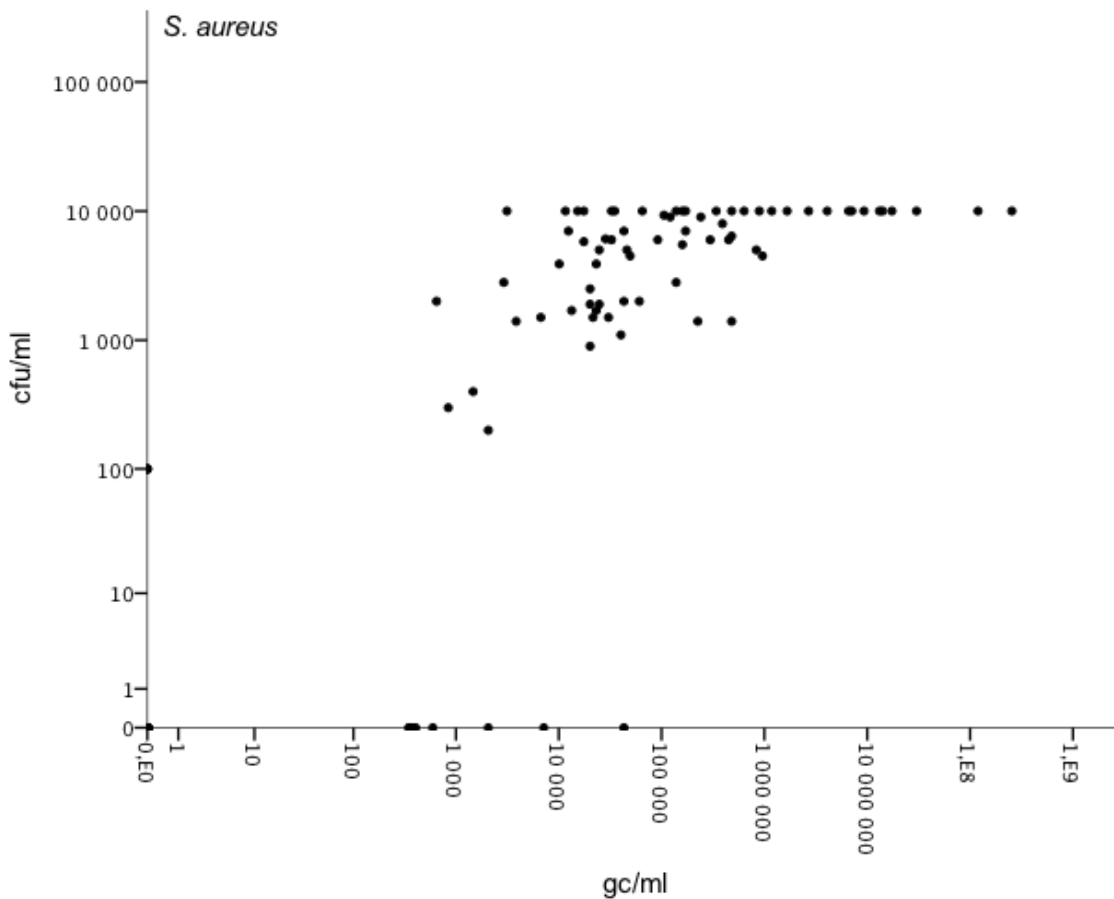


Fig 6. Scatterplot of all samples positive for *S. aureus* with either PCR (gc/ml) or BC (cfu/ml) or both from study I (n=81). Bacteria counts exceeding 100 cfu on agar plates were recorded as 100, which explains the accumulation of the *S. aureus* on the high Y-axis. Ct cut-off for a positive PCR result was 37.0 and a cut-off for considering a sample positive for *S. aureus* in BC was 100 cfu/ml.

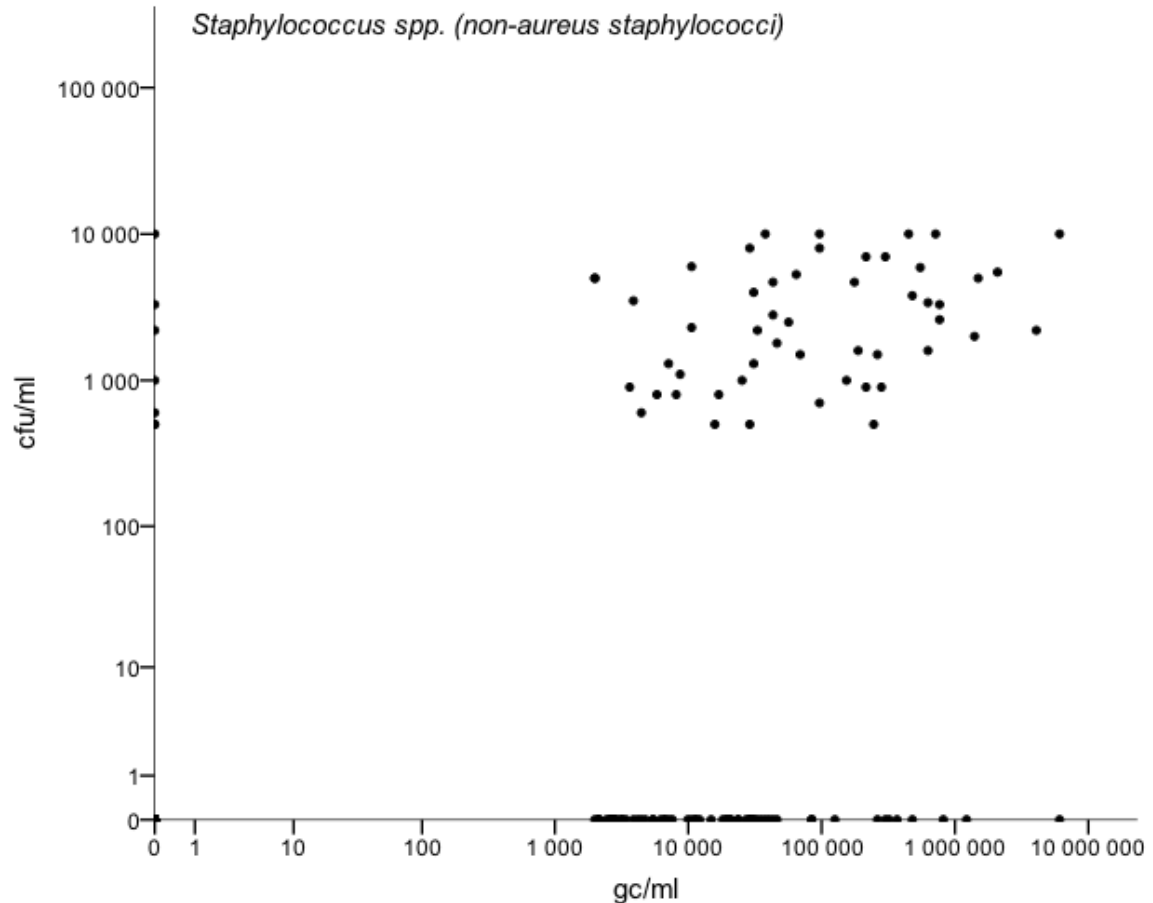


Fig 7. Scatterplot of all samples positive for *Staphylococcus spp. (non-aureus detections)* with either PCR (gc/ml) or BC (cfu/ml) or both from study I (n=121). Ct cut-off for a positive PCR result was 37.0 and a cut-off for considering a sample positive for *Staphylococcus spp.* in BC was 500 cfu/ml.

In the experimental study (IV), correlation between cfu/ml and gc/ml for *Staphylococcus spp.* for the whole 336 h period was 0.55. For the first 36 h, the subsequent 45 – 168 h and 240 – 336 h PC the corresponding figures were 0.68, 0.44 and 0.46. Correlation between cfu/ml and gc/ml varied according to the species and the cow. For *S. epidermidis*, the correlation coefficient was 0.60 (Fig 8, Panel A) and for *S. simulans* 0.55 (Fig 8, Panel B). In *S. epidermidis* infected quarters (Cows 1 - 8) correlations were 0.80, 0.35, 0.70, 0.59, 0.70, 0.81, 0.31 and 0.69. For quarters infected with *S. simulans* (Cows 1 - 2 and 4 - 8) correlations were 0.67, 0.75, 0.66, 0.57, 0.55 and 0.85 (IV).

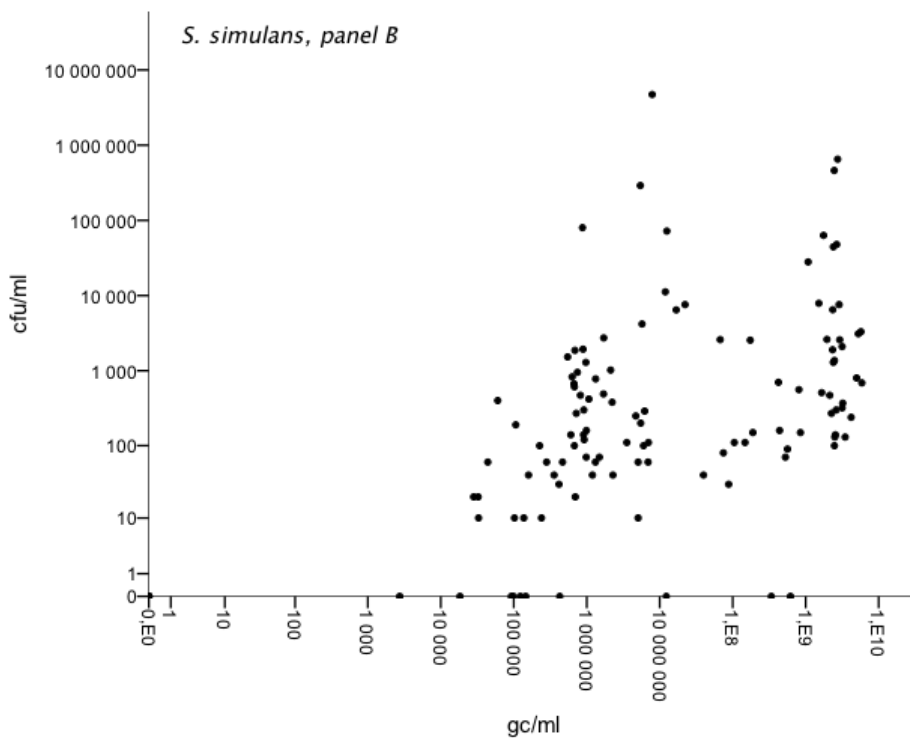
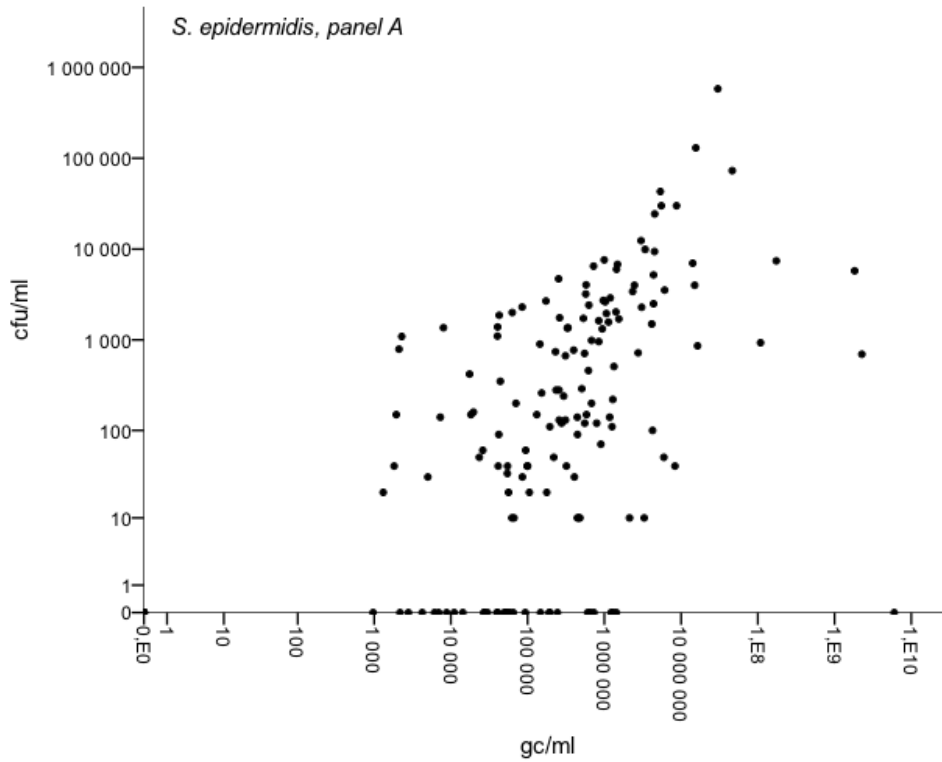


Fig 8. Scatterplot of colony forming units (cfu/ml) from culture results for *S. epidermidis* (n=160, panel A) and *S. simulans* (n=120, panel B) and genome copy (gc/ml) results from PCR for *Staphylococcus* spp. (non-aureus staphylococci) in the experimental study IV. The high bacterial counts in BC were enabled by counting the colonies from several serial dilutions per sample.

5.1.5. Elimination of bacteria according to PCR and BC (IV)

Elimination of experimentally induced bacteria from a mammary quarter was followed with BC and PCR in study IV. Bacterial count curves behaved similarly according to both diagnostic methods during the acute phase, i.e. for the first 36 h. In the subsequent phase, 45 – 168 h, the number of gc/ml increased again and exceeded the highest numbers recorded during the acute phase. Colony counts from culture also increased after the first 36 h, but never reached the numbers recorded during the acute phase (Fig 9). Bacterial counts with both methods, BC and PCR, were lower in samples defined as cured at the end of the study period compared with those defined as persistent (IV) (Fig 9).

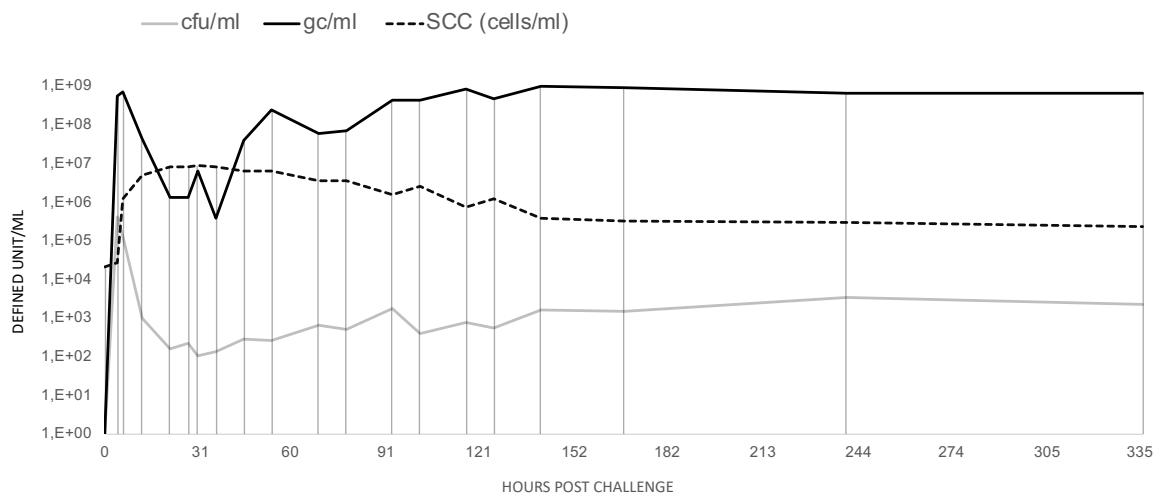
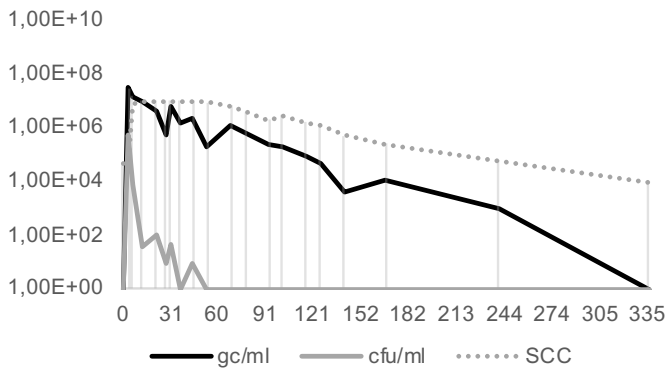


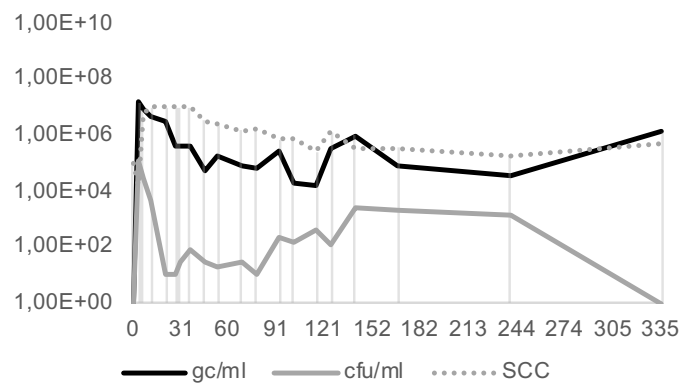
Fig 9. Mean bacterial counts from conventional culture (cfu/ml), genome copy numbers (gc/ml) from multiplex real-time PCR and milk SCC (cells/ml) in mammary quarters (n=14) experimentally infected with *Staphylococcus* spp. during the two-week (336 h) study period presented on a logarithmic scale. Vertical lines show time points of milk sampling. Acute phase of the infection was defined as the first 36 h post challenge, the subsequent phase up to one week (45 - 168 h) and the second week including samplings at 240 and 336 h PC.

Four quarters were defined cured at the end of the study period according to BC results. Only one of those quarters was defined as being negative in PCR (Fig 10, Panel a). Negative culture samples, indicating bacterial cure of IMI, were seen in BC several days before negative PCR results (Fig 10, Panels a, c and d).

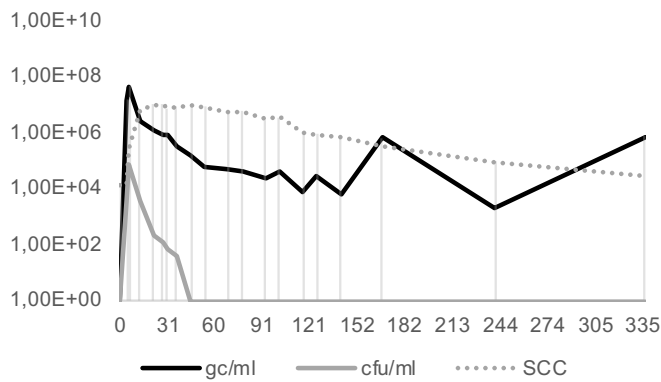
A: Cow 1, *S. epidermidis*



B: Cow 2, *S. epidermidis*



C: Cow 6, *S. epidermidis*



D: Cow 6, *S. simulans*

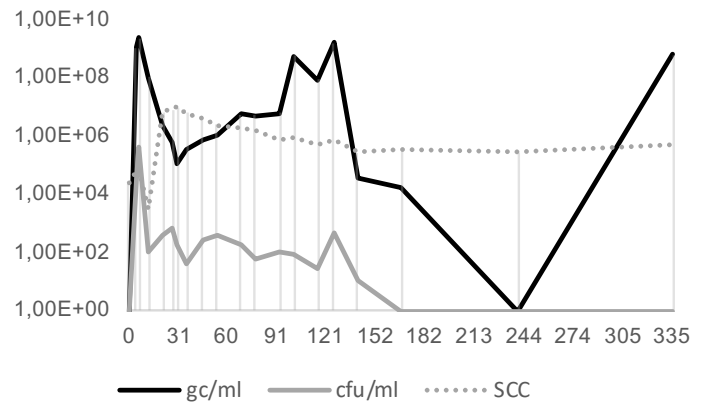


Fig 10. Panels A - D show elimination of *Staphylococcus* spp. from individual quarters with bacteriological cure during the two-week study period (336 h). Definition for cure was a negative culture result (<10 cfu/ml) in the last sampling at 336 h post challenge. Bacterial counts were assessed with conventional bacterial culture (cfu/ml) and with multiplex real-time PCR (genome copies; gc/ml). Three of the quarters were infected with *S. epidermidis* (Panels A - C) and one with *S. simulans* (Panel D). Milk SCC of the quarters is also shown. Vertical lines in panels show milk sampling time points.

5.2. The effect of milk sampling technique on multiplex real-time PCR results (II, III)

5.2.1. The effect of sampling method on the number of species per sample

The number of PCR negative samples was higher in samples taken with experimental techniques than using conventional aseptic sampling ($p < 0.05$, Table 10). The number of species detected per sample was significantly lower in samples taken with cannula or needle compared with the conventional samples ($p < 0.05$, Table 10). From samples collected with the experimental needle method, 69.9%, 68.1% and 46.9% of samples yielded a positive DNA finding with Ct cut-off values of 40.0, 37.0 and 34.0 (II). Of the samples taken with a cannula, 74.5% were positive with the routine cut-off of 37.0 (III) (Table 10).

The number of multispecies samples among conventionally taken samples with a Ct cut-off of 37.0 was 9% (10/113) in study II and 21% (31/149) in study III (Table 10). In samples taken by needle only 1% (1/113) included more than two different pathogens. In samples taken with a cannula, 5% (8/149) included more than two different species.

In multispecies samples obtained with conventional milk sampling, *Staphylococcus* spp. and *C. bovis* were present in all samples with Ct cut-offs of 40.0 and 37.0 (II). These two represented also the most prevalent findings for multispecies, conventionally taken samples of study III.

Table 10. Number of samples containing 0 - 5 different target species per sample in studies II and III, where experimental needle and cannula sampling methods were compared with the conventional aseptic milk sampling techniques. Results of study II are presented with three different Ct cut-offs, 40.0, 37.0 and 34.0, and results of study III with a Ct cut-off of 37.0. Total number of sampled quarters was 113 in study II and 149 in study III. All samples presented here were examined with PathoProof™ Complete-16 Kit. N¹ is the total number of different target pathogens in a sample.

N ¹	Study II, Conventional			Study II, Needle			Study III, Conventional	Study III, Cannula
	Ct 40.0	Ct 37.0	Ct 34.0	Ct 40.0	Ct 37.0	Ct 34.0	Ct 37.0	Ct 37.0
0	26	26	45	34	36	60	27	38
1	31	37	40	55	61	47	59	74
2	40	40	25	21	15	6	32	29
3	12	9	3	3	1	0	21	4
4	2	1	0	0	0	0	10	4
5	2	0	0	0	0	0	0	0
Total	113	113	113	113	113	113	149	149

5.2.2. The effect of sampling method on the detected bacterial species

The most prevalent pathogens in samples taken with the conventional aseptic milk sampling technique in study II were *Staphylococcus* spp. and *C. bovis*, followed by yeasts and *Str. uberis* (II). With needle sampling, the most frequent pathogens were *C. bovis*, *Staphylococcus* spp., yeasts and *Str. uberis* (II).

The number of positive *Staphylococcus* spp. detections in conventionally taken milk samples were over twice as high as in samples obtained with the needle method ($p < 0.05$, II) (Fig 11). For pathogens other than *Staphylococcus* spp. and *C. bovis*, the number of positive samples was too low for statistical comparisons to be made.

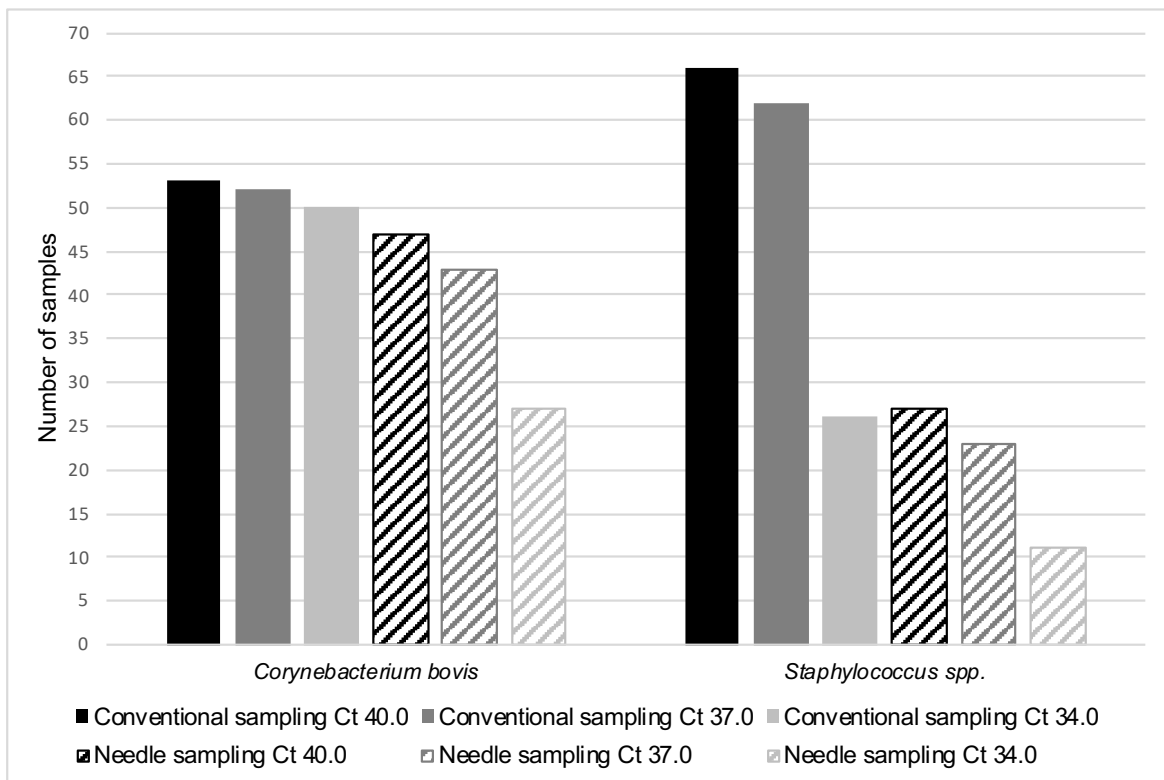


Fig 11. Number of samples positive for *Corynebacterium bovis* and *Staphylococcus* spp. (non-aureus staphylococci) taken using the conventional milk sampling technique and using the experimental needle technique. Results are presented for three different Cycle threshold (Ct) cut-offs (40.0, 37.0, and 34.0).

In study III, the most prevalent species in conventionally taken samples were *Staphylococcus* spp. and *C. bovis*, followed by yeasts and *Str. dysgalactiae* (Table 13).

Samples taken with cannula most often included DNA from *Staphylococcus* spp., *C. bovis*, *S. aureus* and yeasts (Table 13).

Staphylococcus spp., yeasts, *T. pyogenes*/*P. indolicus* and *Str. dysgalactiae* were detected more often in conventional samples compared with cannula sampling (III, $p < 0.05$). The amount of amplified DNA for *Staphylococcus* spp., *S. aureus*, *C. bovis*, *T. pyogenes*/*P. indolicus* and *Str. dysgalactiae* was also significantly higher in results from conventionally taken samples compared with cannula samples (III, $p < 0.05$).

The sampling technique did not affect the number of samples positive for *C. bovis* or for *S. aureus* DNA, but the amount of DNA of these two pathogens was significantly higher in conventionally taken samples than in cannula samples (III, $p < 0.05$).

5.3. Cycle threshold (Ct) values and the effect of different cut-offs on the PCR results (II-III)

5.3.1. Effect of different Ct cut-off values on the PCR results (II, IV)

The effect of alternative Ct cut-off values on PCR results were observed in studies II and IV. Ct cut-off values had a significant effect on the number of positive samples in both studies ($p < 0.05$). In conventionally taken samples, the percentage of the samples positive for target-DNA was 77% with Ct cut-offs of 40.0 and 37.0 and 60.2% with a cut-off of 34.0 (II). Lowering the Ct cut-off from 40.0 to 34.0 decreased the number of positive *Staphylococcus* spp. results by over 50% in study II with both sampling methods.

In study II, the number of negative samples for target DNA in conventionally sampled quarters doubled when the Ct value was lowered from 40.0 and 37.0 to 34.0 (Table 10). The number of PCR-negative samples taken with the needle method increased from 30% (Ct 40.0) and 32% (Ct 37.0) to 53% with Ct cut-off 34.0 (Table 10).

Proportion of samples taken with conventional methods including more than two different species was 14.2% with a Ct cut-off of 40.0, 9% with Ct of 37.0 and 2.7% with Ct cut-off of 34.0. In samples taken by needle, the proportion of samples with more than two target species was 3% with Ct cut-offs of 40.0 and 37.0 and 1% with a Ct cut-off of 34.0 (Table 10).

In study II, the most substantial change ($p < 0.05$) at species level was seen with the proportion of *Staphylococcus* spp. when results were considered with a Ct cut-off of 34.0 instead of 37.0 (Fig 11). The number of major pathogen found was too small for statistical calculations, but in descriptive data the change of Ct value from 37.0 to 34.0 did not seem to change the results.

Table 11. The number of targeted DNA results from quarter milk samples examined with multiplex real-time PCR (PathoProof™ Complete-16 Kit). Quarters were sampled using an aseptic conventional milk sampling technique and an experimental needle sampling technique and observed with three different Ct cut-offs: 40.0, 37.0 and 34.0 (II). All samples were negative for *Klebsiella* spp., *Serratia marcescens* and *Mycoplasma bovis*.

Target	Conventional sampling			Needle sampling		
	Ct 40.0	Ct 37.0	Ct 34.0	Ct 40.0	Ct 37.0	Ct 34.0
<i>blaZ</i>	13	10	5	12	12	5
<i>Corynebacterium bovis</i>	53	52	50	47	43	27
<i>Enterococcus</i> spp.	4	4	3	4	3	3
<i>Escherichia coli</i>	1	0	0	1	0	0
<i>Mycoplasma</i> spp.	1	1	0	0	0	0
<i>Prototheca</i>	1	1	1	2	1	1
<i>Staphylococcus aureus</i>	4	4	4	4	4	4
<i>Staphylococcus</i> spp.	66	62	26	27	23	11
<i>Streptococcus agalactiae</i>	0	0	0	1	1	0
<i>Streptococcus dysgalactiae</i>	3	3	2	2	2	2
<i>Streptococcus uberis</i>	12	12	11	8	8	8
<i>Trueperella pyogenes/ Peptoniphilus indolicus</i>	3	1	1	1	1	1
Yeast	17	9	1	9	8	2
Number of negative samples	26	26	45	34	36	60
Total number of samples	113	113	113	113	113	113

In the experimental challenge (IV), 94% (264/280), 90% (252/280) and 80% (224/280) of the samples were positive for *Staphylococcus* spp. DNA with Ct cut-offs 40.0, 37.0 and 34.0.

The only quarter defined as cured with PCR (negative in the last sampling) was negative at 336 h PC with Ct 40.0, but with a Ct cut-off 37.0 this IMI would have been considered cured at 240 h PC and even at 126 h PC if a tighter cut-off (34.0) was used (Table 12). Observing the results with a cut-off of 37.0, a total of six quarters had one or several negative PCR results during the sampling period. When using the lower cut-off of 34.0, some samples of nine different quarters would have yielded negative results during study periods (Table 12). Of the total number of culture negative samples, 63% (Ct 37.0) and 43% (34.0) yielded positive PCR results for *Staphylococcus* spp., with most of these samples occurring between 45 – 336 h PC. Bacterial counts varied considerable compared with genome copy numbers from PCR. Some of the sample culture negative samples gave low Ct values in PCR, indicating high amount of bacterial DNA in the sample and vice versa (IV).

Table 12. Ct values of the quarters experimentally infected with *S. epidermidis* (n=8) and *S. simulans* (n=6). A quarter was defined cured in bacterial culture if it was negative (<10 cfu/ml) in the final sampling at 336 h post challenge. No DNA was detected in negative (neg) multiplex real-time PCR results with highest possible Ct cut-off (40.0). Sampling results highlighted in dark grey were negative when considered at a PCR Ct cut-off of 37.0. Additionally, the negative results with a Ct cut-off of 34.0 are highlighted with lighter grey. PC = hours post challenge.

Hours PC	Ct values, <i>S. epidermidis</i>							Ct values, <i>S. simulans</i>						
	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5	Cow 6	Cow 7	Cow 8	Cow 1	Cow 2	Cow 5	Cow 6	Cow 7	Cow 8
0	neg	neg	neg	neg	neg	neg	16.6	neg	neg	neg	neg	neg	neg	neg
4	24.5	25.5	neg	33.7	30.1	25.5	27.9	29.3	29.8	26.5	18.0	19.1	17.9	18.6
6	25.6	26.3	37.5	27.0	27.1	23.8	27.3	27.3	27.0	25.8	18.4	17.9	17.7	17.8
12	26.4	27.4	31.8	32.0	28.9	28.2	32.6	35.0	26.7	28.4	24.1	22.6	29.2	20.5
21	27.4	27.8	31.7	29.3	30.1	29.2	32.4	30.8	30.3	29.7	29.0	28.4	29.6	30.2
27	30.7	30.7	39.5	31.5	31.6	29.9	33.5	31.8	29.7	27.3	29.7	30.2	26.8	28.3
30	26.9	30.9	33.9	30.4	29.2	29.7	33.1	32.9	31.8	29.9	23.1	32.9	31.5	29.6
36	29.0	30.8	33.6	31.3	30.5	31.3	33.9	34.3	30.7	30.2	30.9	31.1	34.2	31.7
45	28.4	33.9	37.2	30.6	30.8	32.4	22.6	29.8	30.1	30.0	20.5	30.1	33.1	26.7
54	32.0	32.2	35.9	29.4	30.3	33.8	33.7	38.9	32.7	30.3	17.4	29.5	25.8	33.0
69	29.3	33.3	35.4	31.2	27.4	33.9	34.0	38.7	32.3	27.0	19.6	27.0	27.1	27.7
78	30.3	33.7	39.0	29.8	29.1	34.4	33.0	30.5	32.5	28.8	19.5	27.1	33.0	22.1
93	31.7	31.4	35.2	29.0	28.3	35.0	31.6	29.1	32.5	25.9	17.5	26.9	20.9	17.9
102	32.0	35.6	34.8	32.5	28.1	34.4	34.3	32.2	30.8	29.2	17.9	20.1	20.2	17.8
117	33.1	35.6	33.0	31.6	30.1	36.7	36.9	27.9	34.7	29.6	18.0	22.9	17.1	16.8
126	34.1	31.3	38.4	31.2	30.5	35.0	38.8	36.8	34.9	30.1	17.8	18.5	21.7	18.1
141	37.8	29.6	34.2	30.4	29.5	37.0	26.9	31.6	29.3	29.7	17.9	34.7	16.8	16.6
168	36.3	33.3	34.4	29.5	25.4	30.2	18.0	27.4	28.8	25.3	18.0	35.6	17.9	16.6
240	40.0	34.4	29.6	29.0	18.3	38.7	21.9	30.4	33.8	23.3	17.7	38.4	17.9	17.5
336	neg	29.2	27.7	29.7	31.0	30.0	30.3	34.3	30.5	24.9	17.6	20.0	17.5	18.2
Cure ¹	yes	yes	no	no	no	yes	no	no	no	no	no	yes	no	no
	at	at				at						at		
	54h	336				45h						168		
	PC	h				PC						h		
		PC										PC		

¹Defined cured in culture i.e. 0 cfu/ml in last sampling

5.3.2. Ct values

Species present in samples with the highest Ct values in conventionally sampled results were yeasts and *Staphylococcus* spp. (I-III) (Table 13), indicating the lowest amounts of detected DNA. Species present with the lowest average Ct values were *Str. dysgalactiae*, *S. aureus* and *S. uberis*, indicating the highest amounts of detected DNA (Table 13).

In results originating from experimentally sampled quarters, the results were similar, yeasts and *Staphylococcus* spp. representing the highest average Ct values, followed by *C. bovis* (Table 10). *Str. dysgalactiae*, *Str. uberis* and *S. aureus* were present with the lowest average Ct values, indicating high amounts of DNA (Table 14).

Table 13. Number of positive samples (N) and Ct values (median, mean, SD) of targeted pathogens from studies I-III. All the samples originated from natural IMI and were sampled using an aseptic conventional milk sampling technique. Samples from study I were examined with PathoProof™ Complete-12 Kit and samples from studies II and III with Complete-16 Kit, which included additional targets for *Mycoplasma* spp., *M. bovis*, *Prototheca* spp. and yeasts. All the results are presented with Ct cut-off 37.0.

	Study I				Study II				Study III			
	N	MEDIAN	MEAN	SD	N	MEDIAN	MEAN	SD	N	MEDIAN	MEAN	SD
<i>BlaZ</i>	-	-	-	-	10	33,8	31,8	5,1	27	33,0	32,2	3,4
<i>Corynebacterium bovis</i>	28	32,3	32,3	2,9	52	28,9	29,2	2,1	26	31,5	30,7	2,6
<i>Enterococcus</i> spp.	7	30,7	31,1	3,7	4	32,0	32,3	3,3	5	30,0	31,3	4,3
<i>Escherichia coli</i>	17	29,2	30,6	4,8	0	-	-	-	14	27,6	28,7	5,2
<i>Klebsiella</i> spp.	6	33,9	28,6	9,5	0	-	-	-	5	34,4	31,2	8,4
<i>Mycoplasma bovis</i>	0	-	-	-	0	-	-	-	0	-	-	-
<i>Mycoplasma</i> spp.	0	-	-	-	1	36,5	36,5	-	0	-	-	-
<i>Prototheca</i>	0	-	-	-	1	25,7	25,7	-	2	35,5	35,5	2,0
<i>Serratia marcescens</i>	1	35,0	35,0	-	0	-	-	-	0	-	-	-
<i>Staphylococcus aureus</i>	77	29,7	28,8	4,4	4	27,0	27,2	1,3	19	28,1	27,3	5,8
<i>Staphylococcus</i> spp.	114	33,1	32,9	3,1	62	34,7	33,5	3,3	69	32,0	31,4	3,9
<i>Streptococcus agalactiae</i>	1	22,7	22,7	-	0	-	-	-	0	-	-	-
<i>Streptococcus dysgalactiae</i>	39	28,1	27,9	4,2	3	23,6	25,5	9,4	23	32,2	30,4	5,7
<i>Streptococcus uberis</i>	38	29,2	29,3	5,2	12	30,4	30,1	3,4	18	29,9	29,9	5,0
<i>Trueperella pyogenes/</i> <i>Peptoniphilus indolicus</i>	29	31,2	30,1	6,6	1	21,9	21,9	-	17	35,1	31,4	6,2
Yeast	0	-	-	-	9	36,2	35,5	2,1	35	34,4	33,8	2,4

Table 14. Number of positive samples (N) and Ct values (median, mean and SD) of the detected target pathogens and *blaZ* (PathoProof™ Complete-16 Kit) from milk samples from studies II and III. All the samples originated from natural IMI. Samples were taken using experimental needle (II) or cannula (III) techniques. All the results have been presented here with a Ct cut-off of 37.0.

	Needle, study II, n=113				Cannula, study III, n=149			
	N	MEDIAN	MEAN	SD	N	MEDIAN	MEAN	SD
<i>BlaZ</i>	12	35,2	32,9	4,0	19	34,8	33,0	3,4
<i>Corynebacterium bovis</i>	43	33,1	33,0	2,2	23	33,6	33,0	2,6
<i>Enterococcus</i> spp.	0	-	-	-	5	32,7	32,7	3,9
<i>Escherichia coli</i>	3	30,4	30,0	1,1	9	26,1	26,3	2,9
<i>Klebsiella</i> spp.	0	-	-	-	3	33,1	27,9	9,7
<i>Mycoplasma bovis</i>	0	-	-	-	0	-	-	-
<i>Mycoplasma</i> spp.	0	-	-	-	1	36,4	36,4	-
<i>Prototheca</i>	1	26,8	26,8	-	0	-	-	-
<i>Serratia marcescens</i>	0	-	-	-	0	-	-	-
<i>Staphylococcus aureus</i>	4	28,5	28,5	1,2	17	31,0	30,0	4,8
<i>Staphylococcus</i> spp.	23	35,1	32,7	4,3	56	33,6	32,9	3,0
<i>Streptococcus agalactiae</i>	1	34,8	34,8	-	0	-	-	-

<i>Streptococcus dysgalactiae</i>	2	23,5	23,5	1,6	11	27,9	28,3	4,7
<i>Streptococcus uberis</i>	8	29,2	27,9	4,1	14	32,5	31,6	5,4
<i>Trueperella pyogenes/ Peptoniphilus indolicus</i>	1	23,8	23,8	-	6	30,0	29,7	6,2
<i>Yeast</i>	8	35,4	34,4	2,5	15	35,0	34,3	2,2

5.4. Indicators of inflammation (I-IV)

5.4.1. NAGase (I)

Mean Nagase activity for the samples in study I was 5.79 pmol 4-MU/min/ μ l (I), and 1.04 pmol 4-MU/min/ μ l is considered the upper limit for normal milk (Hovinen et al., 2016). According to the NAGase activities, 246/294 (83.7%) of the samples originated from mastitic quarters.

5.4.2. SCC (II, III, IV)

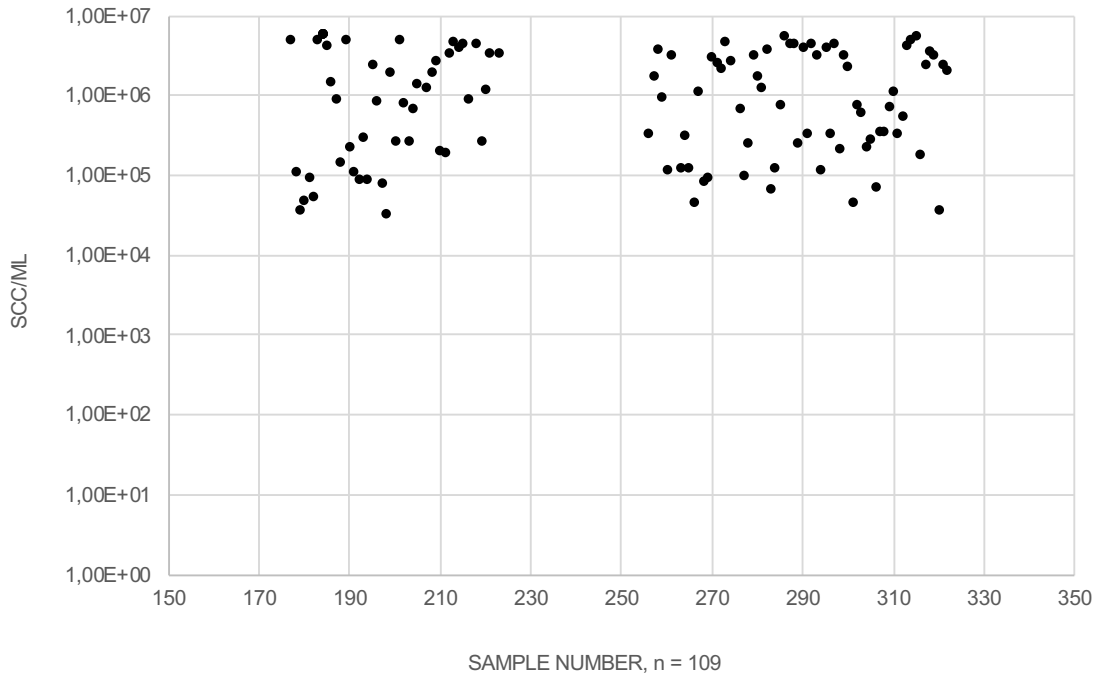
Mean SCC for 109 (of the 113 samples) in study II was $1\,748 \times 10^3$ cells/ml with a range from 32 to $5\,722 \times 10^3$ cells/ml and a median of 901×10^3 cells/ml of milk. The remaining 4 of the 113 clinical mastitis samples gave an error in the DCC device because of clotting of the sample and were not included. SCC was over $1\,000 \times 10^3$ cells/ml in 49% (53/109) of the samples and below 100×10^3 cells/ml in 15% (16/109, II) of the samples. In Fig. 12, panel A, the samples form two clusters, because the samples are arranged according to their original sampling numbers, the results on left cluster is from herd 1 and the other cluster includes the results of the herd 2 (Fig 12, panel A).

In study III, 59/149 (39.6%) milk samples were collected from quarters with clinical mastitis, but milk SCC could not be determined from 22/59 clinical mastitis samples because of flakes and clots in the milk. Thus, a total of 37/149 (24.8%) samples were included in the analysis. The mean SCC was $3\,239 \times 10^3$ cells/ml (SD $1\,705 \times 10^3$ cells/ml) and median $3\,198 \times 10^3$ cells/ml. A total of 90/149 (60.4%) samples originated from quarters with subclinical mastitis with mean SCC of $2\,043 \times 10^3$ cells/ml (SD $1\,432 \times 10^3$ cells/ml) and median $2\,071 \times 10^3$ cells/ml (Fig 12, panel B.)

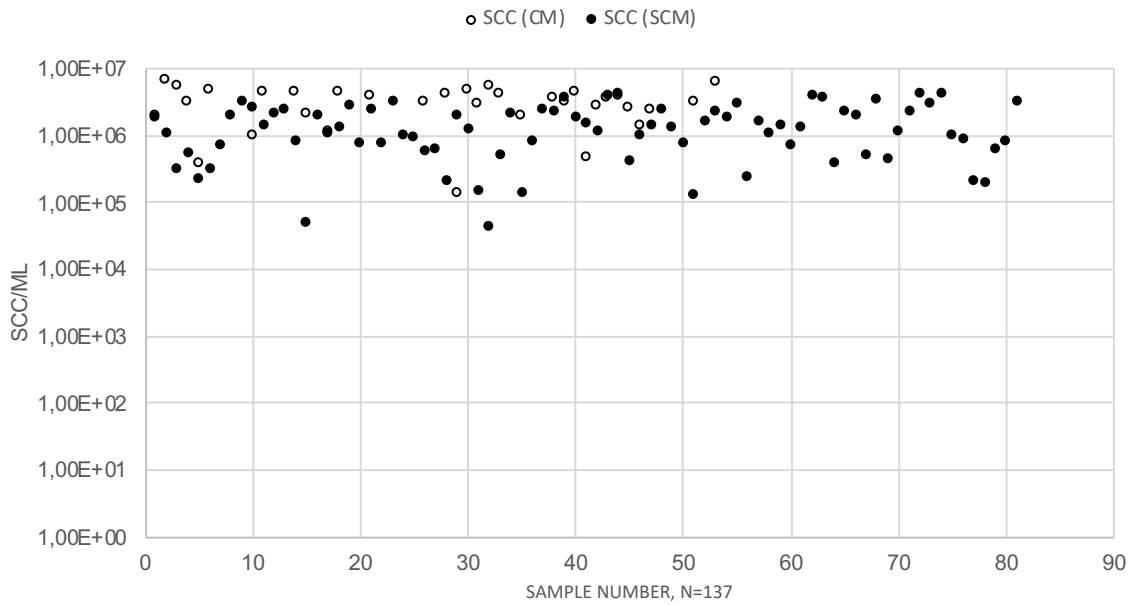
In study IV, SCC was measured at every sampling point resulting a total of fourteen SCC recordings for each quarter (Fig 12, panel C). SCC exceeded 100×10^3 cells/ml in all infected quarters during the study period. Levels of SCC reaching the upper detection limit ($10\,000 \times 10^3$ cells/ml) were recorded between 12 - 45 h post challenge (PC), except for one quarter (IV). At the end of the two-week study period, SCC of two quarters infected with *S.*

epidermidis and three quarters infected with *S. simulans* had decreased to under $< 100 \times 10^3$ cells/ml. One of these quarters was also defined as being bacteriologically cured (IV).

SCC, panel A



SCC, panel B



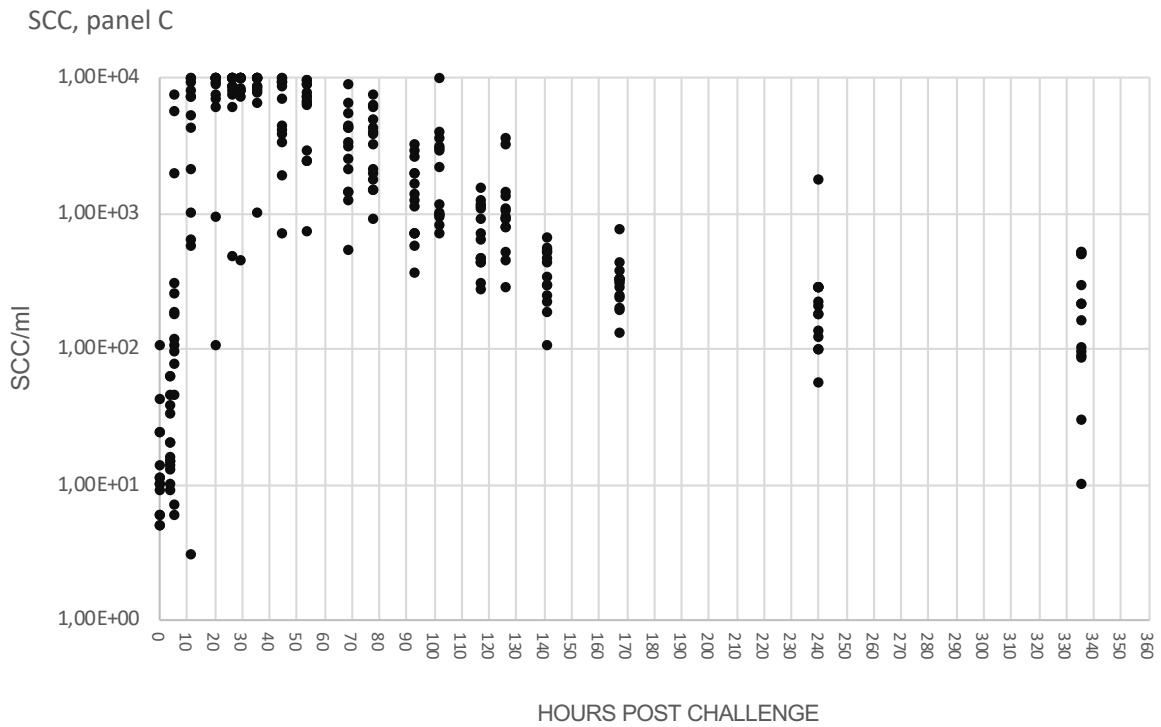


Fig 12. SCC for studies II (panel A), III (panel B) and IV (panel C) are presented. SCC from study II are sorted according to sampling number, which leads to two different clusters seen on the figure (panel A). Results from study III (panel B) are randomly allocated, but SCC of quarters diagnosed with subclinical mastitis and clinical mastitis are presented separately. SCC from study IV is sorted according to the sampling time points resulting a total of 14 SCC recordings per sampling point. All results are presented on a logarithmic scale in cells/ml.

6 Discussion

6.1. Comparison of multiplex real-time PCR and conventional culture

Bacterial culture has been the method of choice in bovine mastitis diagnostics, but the existence and usefulness of DNA-based molecular identification protocols have recently been acknowledged (Middleton et al., 2017). Differences between multiplex real-time PCR and BC as diagnostic tests are substantial. The basic principle in BC is to grow living microbes from the sample in favourable conditions, evaluate bacterial abundance by assessing the extent of bacterial colonies if possible, and identify the organisms by evaluating their phenotypic features. In multiplex real-time PCR, the principle is to extract all DNA from the sample, amplify pre-determined target sequences from the microbial genomes present and evaluate the bacterial counts based on detection limits (Ct values) or numbers of genome copies. DNA is amplified in PCR irrespective of the viability or original fitness of the microbes. When a new diagnostic method becomes available it must be compared with the standard method in order to understand it and put it into context for routine use. The methodology in culture and the PCR test panels play important roles when comparing BC and PCR results and must be taken into account when literature is evaluated.

In the first study included in this thesis we studied quarter milk samples taken from naturally occurring mastitis cases. In contrast with studies published to date, the proportion of microbiologically positive samples was lower when examined with PCR (83%) than with traditional culture (85%, I). In previous studies, milk samples originating from clinical (Koskinen et al., 2010; Keane et al., 2013) or subclinical (Koskinen et al., 2010; Spittel and Hoedemaker, 2012) spontaneous naturally occurring mastitis were more often positive for udder pathogens when tested with PCR than with BC. The discrepancy between the results is likely explained by the in-depth culture method we used, where each morphologically different pathogen on agar plates was enumerated and identified. This level of species identification in BC needs high levels of expertise and advanced culture protocols and agars for rare pathogens, and results in increased costs. In routine mastitis laboratories, basic identification schemes have been considered sufficient (Middleton et al., 2017). The PCR test panel used in study I and in the other studies referred to, included only 11 target pathogens (PathoProof™ Complete-12 Kit), which raised concern as to whether the spectrum of the panel was sufficient for routine, quarter-based mastitis diagnostics.

In 26% (77/294) of the samples from study I, species not included in the PCR test panel were cultured with a detection limit of 100 cfu/ml. The most prevalent species not detectable with PCR were *Corynebacterium* spp. other than *C. bovis*, *Aerococcus* spp. and *Bacillus* spp., all very uncommon mastitis-causing pathogens, at least in the latest culture-based survey carried out in Finland (Pitkälä et al., 2004). If the detection limit for BC was increased to 500 cfu/ml, the proportion of samples with pathogens not detectable by the PCR panel decreased to 2.4% (7/294). Some of these IMI, not detectable with PCR, were

caused by yeasts, which belong to the current test panel (PathoProof™ Complete-16 Kit). In a German study, only 1% (7/681) of quarter milk samples from subclinical mastitis cases included species outside the target panel: one *Pseudomonas* spp. and six yeasts (Spittel and Hoedemaker, 2012). Keane et al. (2013) concluded that the PCR assay targeted all the major pathogens associated with clinical mastitis in Ireland, reflecting well the pathogen profile in the Irish population defined previously using culture techniques. The same PCR assay was used in both of these studies. According to our results and the associated literature it seems that the current PCR panel in use, with additional oligos for *Mycoplasma* spp., *Mycoplasma bovis*, *Prototheca* spp. and yeasts (PathoProof™ Complete-16 Kit), is sufficient for diagnosing bovine IMI from quarter milk samples.

Culture-negative samples from cases of clinical and subclinical mastitis have been a common finding in BC, causing frustration to veterinarians and dairy producers. Using a PCR assay, many of these samples have contained DNA of target pathogens. Proportions of target-DNA positive results from culture-negative samples (BC-/PCR+) have varied from 43% (Taponen et al., 2009) and 47% (Bexiga et al. 2011a) up to 79% (Keane et al., 2013). Our results from naturally occurring IMI (I) were similar: 47% of the culture negative samples were positive for target-DNA in PCR. In our study, the detected amount of DNA in all these samples was low, which may indicate contamination or already clearing infection (I). Our results agree with those of Spittel and Hoedemaker (2012), who reported that amount of DNA was usually high if the species was detected in the sample with both diagnostic methods, BC and PCR. For example, for *S. aureus*, the mean Ct was 29.4 in BC+/PCR+ samples compared with 34.0 in PCR+/BC- samples (Spittel and Hoedemaker, 2012), which indicates almost 14 times as high amounts of DNA in PCR+/BC+ samples as in PCR+/BC-. In our studies (I-III), *S. aureus* was present with mean Ct values ranging from 27.7 to 28.8 (Table 13), but in culture negative samples in study I, the lowest recorded value was 34.1 (Fig 4). Spittel and Hoedemaker (2012) also presented similar findings with *Staphylococcus* spp. and for *Str. uberis* (Spittel and Hoedemaker, 2012).

Culture-positive results from PCR-negative samples (BC+/PCR-) also occur. This is possible if the organism isolated is not included in the target panel and thus cannot be detected with PCR. In our study I, 27 samples out of 50 PCR- samples (9.2%) were positive in culture. In half of those samples, a species outside of the target panel of PCR was detected. Our numbers were higher compared with previously published papers, with the proportion of BC+/PCR- samples varying between 2% – 7% (Keane et al., 2013; Koskinen et al., 2010; Spittel and Hoedemaker, 2012). The most prevalent species cultured from PCR-negative samples were *Staphylococcus* spp., but only with low bacterial counts, ranging from 100 to 500 cfu/ml. Excessive handling and transportation of the samples in study I may have subjected our samples to cross-contamination, but it does not fully explain the results. Explanation for this could be heterogeneous distribution of microbes in milk samples or poor mixing of the sample, which is doubtful in a standardized reference laboratory. Oligos for commercial PCR assays have been designed specifically to include most common mastitis-causing strains of each target species. It is possible that these *Staphylococcus* spp. species or strains detected in BC were undetectable by the panel we used.

Staphylococcus spp. (NAS) represent a group that stood out on several occasions throughout the work embodied in this thesis. In study I we concluded that PCR was a reliable tool for identification of mastitis-causing *S. aureus*, but it did not perform as reliably with *Staphylococcus* spp. (NAS), using BC as the reference method. The agreement between BC and PCR for detecting *S. aureus* in study I was 0.90 and for NAS 0.44 ($p < 0.05$). Similar observations were published previously (Spittel and Hoedemaker, 2012). In study IV, where only two CNS strains were included, the agreement between BC and PCR was dependent on the timing of the sampling. If the sample was taken during the acute phase of IMI, the agreement between BC and PCR was almost perfect ($\kappa = 0.85$), but decreased to a moderate level as time passed in relation to the challenge. In general, the low agreement between BC and PCR in detecting NAS may be due to the variety of species within the group, with no routine species identification in BC nor in PCR. In study II we showed that up to half of *Staphylococcus* spp. findings in quarter milk samples originated from sites other than the udder. One must also take into account the shortcomings of the reference method, such as different NAS exhibiting different growth capacities in BC.

Differences between *S. aureus* and *Staphylococcus* spp. were observed in the bacterial loads of pathogens detected in the milk samples. In subclinical and clinical cases of spontaneous IMI, *S. aureus* was present usually with low Ct values, indicating high bacterial counts in contrast to *Staphylococcus* spp., which were usually present with high Ct values, indicating low bacterial counts (I). These results agree with those of other studies (Table 15). Higher bacterial counts for *S. aureus* compared with NAS have been reported also in culture-based studies. *S. aureus* has been reported to be shed in milk in natural IMI cases continuously with moderately high colony counts, varying from 8 000 to 338 000 cfu/ml (Walker et al., 2013). In our study (I), the mean cfu/ml for *S. aureus* was 6 000 cfu/ml and for CNS 2 000 cfu/ml (unpublished data). Taponen et al. (2007) reported that bacterial counts in persistent IMI due to NAS were $>1\ 000$ cfu/ml and in transient infections $<1\ 000$ cfu/ml (Taponen et al., 2007). In our experimental study (IV) with high inoculation dose, the bacterial counts were 100 – 400 000 cfu/ml. The feature of *Staphylococcus* spp. usually being present in milk samples at low levels makes it even more difficult to evaluate the clinical relevance of the positive PCR result for *Staphylococcus* spp.

Table 15. Mean Ct values from aseptically collected quarter milk samples for the five most commonly detected target pathogens from our studies and other studies undertaken between 2012 and 2017. All samples were examined with PathoProof™ Complete-12 or Complete-16 Kit.

	<i>S. aureus</i>	<i>Staph. spp.</i>	<i>E. coli</i>	<i>Str. dysgalactiae</i>	<i>Str. uberis</i>	N ³
Study I	28.8	32.9	30.6	27.9	29.3	294
Study II	27.2	33.8	38.4	25.5	30.1	113
Study III	27.4	31.6	28.2	30.0	29.1	149
Spittel et al. 2012 ¹ BC+ PCR+	29.4	30.8	34.2	25.5	26	681
Spittel et al. 2012 BC- PCR+	34	34		33.9	33.9	
Vakkamäki et al. 2017 ²	31.9	31.5	29.3	27.0	26.6	240067

¹subclinical mastitis samples

²samples sent from suspected subclinical and clinical mastitis cases

³total number of analyzed samples in a study

The differences between BC and PCR in diagnosing *S. aureus* and *Staphylococcus* spp. were also evaluated by calculating relative Se and Sp for PCR and testing correlations between cfu/ml and gc/ml (I). Se and Sp for *S. aureus* were 97% and 96% and for *Staphylococcus* spp. 87% and 75%. In comparison, Spittel and Hoedemaker (2012) reported Se and Sp values of 79% and 94% for *S. aureus* and 78% and 68% for *Staphylococcus* spp. Correlation between cfu/ml and gc/ml was higher (0.68) for *S. aureus* than for *Staphylococcus* spp. (0.47) (I). In our experimental study (IV), where healthy quarters were inoculated with high doses of two known CNS species and elimination of bacteria was monitored, correlation between cfu/ml and gc/ml was 0.60 for *S. epidermidis* and 0.55 for *S. simulans* over the whole study period. Correlation between cfu/ml and gc/ml was stronger when only one species and strain was present, which is not surprising. Correlations seemed to vary notably between quarters and cows. This may be due to the host response: if the response is strong, bacteria may not be fit to grow on agar plates, but DNA can remain intact for PCR detection. It was reported in Simojoki et al. (2011) that quarters that cleared the IMI showed a stronger immune response compared with persistently infected quarters (Simojoki et al., 2011). It was also shown that this particular *S. simulans* strain caused a stronger innate immune response than *S. epidermidis* based on higher proinflammatory cytokine levels (Simojoki et al., 2011). Logically, correlations between cfu/ml and gc/ml were poor for cows that were cured or yielded several no-growth results in BC, but continued to be positive in PCR. Comparisons between cfu/ml and gc/ml may be debated because of the basic difference between the units. First, the actual number of bacteria within one colony is not known and can vary among species and strains. Secondly, depending on the microbe and the target gene sequence, several copies of the same target may be

expressed in the same genome (Phuektes et al., 2001; Vetrovsky and Baldrian, 2013). For example, in the 16S rRNA 5.8 ± 2.8 copies per genome were reported for phyla including streptococci, staphylococci, *Klebsiella* spp. and *E. coli* (Vetrovsky and Baldrian, 2013). Whole genomes for different staphylococci are available, but without knowledge of the target sequences in the PCR assays the accuracy of the target sequences cannot be evaluated. Presenting cfu and gc in same unit enabled us to present the elimination curves for CNS in study IV.

PCR methodology lacks definition for a contaminated sample. Currently all detected target pathogens from the sample are reported to the client. In theory, the list may include all fifteen different species. Despite the debated hypotheses about mastitis being related to dysbiosis of the mammary gland (Addis et al., 2016), the current concept remains that one or a maximum of two pathogens cause IMI (Rainard, 2017; Middleton et al., 2017). More than two pathogens in the sample indicate contamination (Middleton et al., 2017). Applying culture-based standards to molecular-based methods has been questioned (Bexiga et al., 2011a; Keane et al., 2013) because quantitative data are available. In commercial multiplex real-time PCR assays, dominance of a target pathogen can be reported if the target gene of some pathogen represents >90 or >99% of all detected DNA in a sample (Thermo Fisher Scientific). A sample result with DNA of a dominant major pathogen, accompanied by small amounts of two minor pathogens, supported by information of clinical signs typical for the major finding, could, in principle, be valid. Such results should, however, be interpreted with caution because detection of more than two bacterial species in a milk sample always raises concerns about the hygienic quality of the sample, despite the method used.

Milk samples for PCR testing are at least as prone to errors in handling and transportation as samples studied with BC. If samples are transported without preservatives or are left uncooled, even low amounts of some pathogens such as *E. coli* could quickly outgrow the other pathogens, resulting a falsely high amount of *E. coli* DNA detected in PCR. A carryover may also be a source of contamination. Mahmmod et al. (2017) studied a DNA carryover in cows being examined with PCR for *S. aureus*. Truly IMI negative cows represent the biggest risk for false positive *S. aureus* diagnosis due to the carryover from an *S. aureus* positive cow milked just before her (Mahmmod et al., 2017). Cross-contamination in laboratories is also a possibility. Despite the risks of contaminated milk samples for the PCR analysis, a high level of hygiene for aseptic quarter milk sampling has been reached in published studies (Table 3). According to the knowledge to date, there is no reason why the concept of contamination could not be the same in PCR as in culture, when examining result sheets for aseptically taken quarter milk samples. All results could still be provided so that no information is lost and also as a feedback to the sampler.

6.2. The effect of milk sampling technique on multiplex real-time PCR results

Aseptic milk sampling has been the basis for generating reliable microbiological results (Middleton et al., 2017), regardless of the diagnostic method. We tested the effect of sampling techniques on PCR results in two different studies (II, III). If the teat canal (III) or the entire teat (II) was bypassed when taking a milk sample, the proportion of PCR negative samples significantly increased. The proportion of multispecies samples (more than two species in a sample) was lower when using experimental techniques compared with samples taken using conventional aseptic sampling techniques. In addition, the amount of target pathogen DNA was higher in samples, which gave positive PCR results despite the sampling methods. If target DNA was present only in conventionally taken sample, the DNA amount was lower. Our results indicate that despite the careful pre-sampling procedures, target DNA originating from outside the mammary gland is often present in conventionally taken samples.

The sampling technique also affected the number of species detected in a sample. The most striking differences were seen with *Staphylococcus* spp. in study II, where the proportion of positive *Staphylococcus* spp. detections was over twofold in conventionally taken samples compared with needle sampling. A higher proportion of *Staphylococcus* spp. positive findings in conventional samples than in cannula samples was also recorded in study III, but the difference was not that notable. In both studies (II, III), the amount of detected *Staphylococcus* spp. DNA was higher in conventional samples than in experimentally taken samples, indicating that staphylococcal DNA originated also from extramammary sites. Some NAS species are common on teat skin, teat canal and orifice (Taponen, 2008). Despite proper cleaning of the teat end, the sterile cannula may be contaminated with target DNA from the teat canal at insertion. In study III, a similar phenomenon was seen also with yeasts and *T. pyogenes*/*P. indolicus*, which were present in conventional samples more than twice as often as in cannula samples. This reflects the characteristics of yeasts as commonly colonizing the barn environment (Radostits et al., 2007) and *T. pyogenes* as an opportunistic pathogen of healthy cows (Madsen et al., 1992).

Our results show that not all pathogen DNA present in milk samples originated from the mammary gland, despite the aseptic sampling protocol. Bacteria and DNA material has been preserved in ethanol for decades but yields of DNA-products amplified from ethanol preserved tissues or bacteria have been low (Barnes et al., 2000; Kilpatrick, 2002). It is not known how the target DNA sequences of PCR mastitis assays behave when subjected to small amounts of 70% alcohol or other disinfectants used in teat end scrubbing. Bacteria are killed, but DNA may stay intact, as DNA tolerates the multiple washing and dilution protocols during DNA extraction before PCR amplification. Use of established pre-milking procedures (discarded foremilk strips and teat end scrubbing with 70% alcohol) improved the sensitivity of PCR for diagnosing *S. aureus* (Mahmmod et al., 2013a), but increased the odds of the sample containing *S. agalactiae* DNA (Mahmmod et al., 2014). The mechanical scrubbing of the teat end to remove pathogens may be more important than the actual

disinfection when PCR is used. Some microbes detected with PCR can be considered as contaminants, especially when present as small amounts of DNA. If the signs of the cow or the degree of udder inflammation do not fit the usual signs caused by the pathogen detected, the possibility of contamination should be considered.

6.3. Cycle threshold (Ct) values and the effect of different cut-offs on the PCR results

The maximum number of cycles in PCR is 40.0 because cycles above 40.0 produces nonspecific amplification products of no value (Mullis et al., 1986; Innis et al., 1990). A generally accepted norm to separate a true positive signal from contamination is three cycles apart from a negative control (Bustin, 2004). This is why the Ct value 37.0 was selected as the standard cut-off for a positive detection in PathoProof™ PCR Assays (Koskinen et al., 2009). Results with Ct values between 37.0 and 40.0 may be of poor reproducibility or reliability (Koskinen et al., 2009). An approximately 3.3 cycle difference in Ct values translates into a ~10-fold difference in the concentration of the target DNA.

The effects of different Ct cut-offs on the interpretation of PCR results were evaluated in study II. The cut-off value had a significant effect on the number of positive samples: the lower the cut-off, the fewer positive samples. Usually the results obtained with cut-offs 40.0 and 37.0 did not differ. This supports the decision to use a Ct cut-off of 37.0, which is adequate for the PCR assay, at least when examining quarter milk samples. The results over 37.0, of questionable reliability, can be neglected. Our results may have been biased due to the presence and prevalence of particular species, because in most of the samples the minor pathogens *Staphylococcus* spp. and *C. bovis* were present (II). Lowering the Ct cut-off value affected most samples with *Staphylococcus* spp. where the number of positive samples decreased by over 50% when the Ct value was decreased from 37.0 to 34.0 (II). Also during study IV, several samples target-positive with a cut-off of 37.0 were negative with a cut-off of 34.0. This effect on the results was seen mostly in samples originating from quarters infected with *S. epidermidis*, but not *S. simulans*. Decreasing the Ct cut-off value from 37.0 to 34.0 did not seem to affect the results (positive IMI or not) regarding major pathogens such as *S. aureus* and *S. uberis*, although we did not have enough data to evaluate these findings statistically (II). Low average Ct values for major pathogens, usually under 30.0 (III), are in agreement with results from a large dataset of Vakkamäki et al. (2017) and support this assumption.

The current practice in some laboratories of presenting Ct results as semi-quantitative classes (high amount of detected pathogen (+++), moderate amount of targeted pathogen (++) or low amount of targeted pathogen (+)) on the report sheet, without providing actual Ct values, could be questioned. In study I, fourteen *S. aureus* positive samples with Ct values between 30.1 – and 37.0, considered low according to the manufacturer, resulted in 2 000 cfu/ml or even 10 000 cfu/ml of *S. aureus* on agar plates, which clearly indicates *S. aureus* IMI (I). Cederlöf et al. (2012) concluded that a Ct cut-off 37.0 was most reliable in

diagnosing *S. aureus* IMI in cows at drying-off compared with Ct cut-offs 32.0, 34.0 and 39.0. Mahmmod et al. (2017) stated that during lactation *S. aureus* should be present in a sample with Ct values under 32.0 to indicate a likely IMI. Both of these studies used cow composite samples, which are diluted, prone to contamination and where Se is lower than in quarter-based samples. We suggest reporting PCR analysis results with actual Ct values, instead of semi-quantitative classes. This would improve the interpretation of the results in routine practice. Until then, we suggest that at least for *S. aureus*, in addition to high (+++) and moderate (++) amounts of detected DNA, also low amounts (+) should be noted. In case of *Staphylococcus* spp. (NAS), results with low amounts (+) should be evaluated with high criticism.

6.4. Evaluation of the inflammatory status of the quarter

Use of NAGase as an indicator of inflammation could be argued against because NAGase has been reported to be inferior compared with SCC for identifying IMI-negative and IMI-positive quarters (Nyman et al., 2016b). On the other hand, it has been reported as an accurate and good measure of udder health by others (Hovinen et al., 2016). NAGase testing protocols and units vary, as reviewed by Hovinen et al. (2016), and it may also be affected by stage of lactation. In study I, milk SCC records were not available. With experience of NAGase testing in our laboratory (Hovinen et al., 2016), NAGase was the best choice as an indicator of inflammation for that study. According to milk NAGase activities, the majority (84%) of study I samples originated from mastitic quarters.

In studies II-IV milk SCC was used as the indicator for inflammation. In study II, we recorded very high SCCs, considering that the most prevalent pathogens detected were *Staphylococcus* spp. and *C. bovis*. One reason behind this could be particularly pathogenic strains present in the study farms, causing strong inflammation and high SCC. Pathogenic characteristics of NAS vary among species (Hyvönen et al., 2009; Supre et al., 2011; Taponen and Pyörälä, 2009). Another reason for high milk SCCs is that both in healthy and subclinically infected quarters, SCC may remain high up to seven hours after milking, with the highest levels recorded soon after milking (Olde Riekerink et al., 2007). Our sampling in study II was carried out within two hours after morning milking, possibly biasing SCC results upwards in the sampled quarters. In study III, sampling took place randomly related to the milkings, the veterinarians taking samples at various times of the day during their visits. Assessment of the type of mastitis, clinical or subclinical, was based on changes in consistency and appearance of the milk and clinical signs of the cow, and not on SCC. Regardless of the type of mastitis, the average SCC of both groups was over 2 000 000 cells/ml, indicating a strong inflammatory reaction. This was expected because a veterinarian had been called to examine and treat these cows for mastitis.

In study IV, because the cows were sampled frequently and always prior to milking, milk SCC was considered to be a reliable inflammation indicator. Mastitis cure can be assessed based on bacteriological criteria (free of pathogens), inflammatory status (quarter

milk SCC < 100 000) and in clinical mastitis, disappearance of clinical signs. We used the bacteriological cure definition because the study period was only two weeks long and the decrease of SCC to a normal level after infection may take longer (Pyörälä, 1988). In cured quarters, SCC appeared to decrease along with gc/ml. Unfortunately, due to the small sample size, we were not able to calculate correlations between SCC and gc/ml. In IMI, the neutrophils are the predominant cell type, ingesting and killing bacteria at the infection site (Rainard and Riollet, 2006). It may be speculated that at least part of the detected DNA could originate from phagocytosed microbial cells. To my knowledge, no studies have reported how long DNA remains intact in white blood cells.

6.5. Limitations of the studies

As evident in this thesis, milk samples are prone to contamination. Samples in study I originated from routine sampling of mastitic cows and were mostly collected by farmers under uncontrolled conditions. A recent study with more than 240 000 quarter milk samples analyzed with PCR, showed, however, that Finnish farmers are well trained in taking milk samples; only 13.4% of the samples included DNA of more than two pathogens (Vakkamäki et al., 2017). In study I, the hygienic quality of samples was even better: 8.1% of the samples studied with PCR included DNA from more than two pathogens and 12.9% was defined as contaminated in BC. In studies II-IV, sampling was carried out by the authors (II, IV) or trained veterinarians and veterinary students (III). In study II the sampling procedure was always the same: foremilk strips and CMT, then a conventional sample and immediately after that the needle sample. In theory, it could be possible that the reduced number of species per needle sample could be partly due to the flushing effect of the conventional samples taken first, if some milk was removed also from the gland cistern. The same sampling order was used also in study III: we took first the conventional samples and then cannula samples. In a study by Bexiga et al. (2011b) a similar sampling protocol with cannula and conventional sampling was used. They used a random sampling order, which had no effect on the results (Bexiga et al., 2011b).

With the sample size in study I, we were able to evaluate the performance of the PCR assay in detecting *S. aureus* and *Staphylococcus* spp. (NAS). In order to get specific information about other species, the study would have benefited from a larger sample size, with power calculations based on the knowledge of the prevalence of different pathogens in Finland (Myllys et al., 1998; Pitkälä et al., 2004). With our in-depth culture method, where each colony from even contaminated agar plates was enumerated and identified, a larger sample size was not possible for economic reasons. In studies II and III, the aim was to compare the effect of the sampling technique on the PCR results. For general comparisons, the sample sizes were adequate, but for pathogen-specific comparisons the studies would have needed more samples. The use of invasive methods (II, III) and challenge of healthy animals (IV) limited the sample sizes in studies II-IV.

Latent Class Analyses (LCA) have increasingly been used in estimations of Se and Sp in the absence of a gold standard (Dohoo et al., 2010). Those models assume that Se and Sp of the diagnostic test should be as good in low prevalence populations as in high prevalence populations and tests should be conditionally independent given the disease status (Dohoo et al., 2010). The models should be tested in two populations of different disease prevalence. Background information on the cows and herds would have increased the validity and reproducibility of study I. Creating groups, e.g. according to breed or region, would have enabled the use of LCA in determining Se and Sp for PCR, instead of using BC as the reference method. This was not possible because the authors were completely uninformed about the origin of the samples. Se and Sp for PCR were probably underestimated, because BC is not a perfect reference.

In study IV, the inoculum volumes of cultured milk (0.1 ml) were higher and definition of a positive result (10 cfu/ml) lower than in routine procedures. This was partly due to the original design for the host-response study (Simojoki et al., 2011), but also because we know that PCR is a very sensitive test which uses 0.35 ml of milk, and we wanted to make the comparison between BC and PCR more fair. If the positive detection limit for CNS had been 500 cfu/ml, as for our national recommendation (Honkanen-Buzalski, 1995), the agreement between BC and PCR would have decreased. Another technical challenge in study IV was that PCR detects *Staphylococcus* spp. in general and not at species level. According to our culture results, no other coagulase-negative staphylococci, nor any other species were present in the samples. Sampling protocol was also strict and the surroundings controlled, but there is no guarantee that a minimal proportion of genome copies detected could have originated from some other NAS species.

6.6. Use of multiplex real-time PCR in bovine mastitis diagnostics – benefits, limitations and future aspects

Use of multiplex real-time PCR in mastitis diagnostics has several benefits, including speed, sensitivity and objectivity. Identification of pathogens according to their genomic sequence is more precise than identification based only on phenotypic expression (Gillespie and Oliver, 2005). In addition, DNA is a highly stable biological molecule (Pereira et al., 2008) that can be amplified from milk samples subjected to antimicrobials or preservatives. The ability of PCR to detect pathogen DNA from growth-inhibited or non-viable cells may be considered an advantage, but is also a disadvantage because the clinical relevance of the result remains open.

All DNA or protein sequence analysis identification systems are based on the assumption that the same species carry unique DNA or protein sequences, which no other species possess (Pereira et al., 2008). Oligo design has focused on highly conserved genomic sequences, but variation in genes is constant due to reproduction, migration and random genetic drift (Pereira et al., 2008), which is why oligos should be monitored over time. Due to delicate processes for producing the best panels and protocols for commercial

products, the oligo sequences are often protected by patents. Consequently, it is impossible to evaluate the test thoroughly or compare it with other tests. Some oligos are designed to detect several species of a microbial group and some oligos are unique for single species, but still able to amplify different strains of the target species. To differentiate *S. aureus* from the group of other staphylococci, the Ct values for detection must differ by at least three cycles. If the design of the oligos is not optimal, no amplification occurs, leading to the possibility of false negative results. Furthermore, the more oligos the panel includes, the more challenging is optimization of annealing conditions, increasing the chance of errors during the cycle (Innis et al., 1990). In addition, there are several kinds of polymerase enzymes, some more prone to errors than others. Of the commercial PCR assays, PathoProof™ PCR Assays use a hot-start polymerase enzyme, which will activate only during the first heating of the first PCR cycle. Information on the type of polymerase enzyme in Mastit 4-panels (DNA diagnostic A/S) was not available, but based on the worksheet guidelines in which all the pipetting is advised to be performed on cooled ice, differences between enzymes may be assumed. Multiplex real-time PCR assays and panels available at present for mastitis diagnostics differ in protocols, test volumes and polymerase enzymes. Independent high quality studies are needed to compare Se and Sp of the different assays.

Finland has excellent BTM SCC figures and high milk quality and Finnish farmers take a lot of quarter milk samples – over 150 000 milk samples are examined only with PCR each year, covering approximately 80% of all samples. In modern large herds, cow level SCC from AMS or DHI samples are used to screen for subclinical mastitis, CMT is performed for high SCC cows and quarter-based samples taken if CMT score is 3–5 on a 5-point scale. Sampling is easy for the farmer: an aseptically taken quarter milk sample is sent to the laboratory in the milk truck or via mail with preservatives, and the result is provided electronically on the same or the following day. Motivation for such monitoring is a prerequisite to maintain good udder health status: information of the most common udder pathogens causing IMI on the farm is used in control plans. With the long withdrawal periods after mastitis treatments in Finland, farmers are willing to take a milk sample from a cow with elevated SCC, hoping that the result does not indicate any need for antimicrobials. PathoProof™ Mastitis PCR was launched in 2008 (Thermo Fisher Scientific, former Finnzymes, Vantaa, Finland) and quickly adopted. The laboratories of the largest dairy company switched from culture to PCR in 2010. Concerns about increasing antimicrobial consumption were raised immediately. Since 2010, however, use of intramammary antibiotics has decreased (Finnish Medicines Agency Fimea, 2017) and the easy logistics has increased use of milk samples in mastitis control. In addition, veterinarians are prohibited by law to profit from selling antimicrobial products. A clear disadvantage of using only PCR at the national level is that bacterial isolates can no more be collected and stored for mastitis surveys and monitoring of resistance. In addition, the only indicator of antimicrobial susceptibility is testing for the presence of the staphylococcal beta-lactamase gene (*blaZ*). Technically, including a target sequence, for example for MRSA, into panels is not difficult, but for a commercial test such changes are corporate decisions, based on economic considerations. In the future, the emerging global antimicrobial resistance

problem may create new rules for treatment protocols, which could also require more efficient monitoring of antimicrobial resistance of udder pathogens.

Using PCR methods, very high analytical sensitivities have been achieved in spiked samples (Graber et al., 2007; Koskinen et al., 2009) as well as in field samples (Cederlöf et al., 2012; Keane et al., 2013; Koskinen et al., 2010). This may partly be due to the sample volume, which is 350 µl in PathoProof™ and 500 µl in Mastit 4 assays, compared with the routine 10 µl for BC. We work in a barn environment full of bacteria, where aseptic milk sampling is a challenge, not to mention samples taken with automatic devices. In these conditions, excessive analytical sensitivity for diagnostic purposes may potentially turn into a disadvantage. Nyman et al. (2016a) concluded that using non-aseptically collected milk samples will increase Se, while Sp is kept at a similar level or lower, compared with BC of aseptically collected milk samples. Mahmmod et al. (2017) concluded that the true status of a cow-based composite milk result studied with PCR, regarding *S. aureus* (Ct values between 32.0 and 37.0), remains “uncertain”. Too high Se in diagnosing mastitis may be problematic, especially for pathogens present at low levels and being common inhabitants of udder skin and the barn environment. Interpretation of composite samples is even more difficult than quarter-based samples. Screening for major and easily spreading pathogens like *M. bovis* from cow composite samples within a herd is economically sensible, but the possibility of carryover when using automatic sampling devices should be taken into account. Mahmmod et al. (2017) suggested that in screening DHI samples with PCR, milking order and the Ct value of the preceding cow should be included in the diagnostic process. BTM samples include a high number of different target pathogens (Katholm et al., 2012), thus their diagnostic value is in country-level screening of rare, highly contagious major pathogens, like *M. bovis* in the Nordic countries. A positive DNA detection from BTM samples may reveal a need for cow level screening to find positive carrier animals.

Clinical relevance is the key point in interpreting PCR results. Bacteriological results must be combined with all available information, including history and clinical signs of the cow, palpatory findings of the udder, changes in milk and SCC. If the signs do not support the microbiological result, it could be questioned. In clear atypical cases, the possibility of contamination or presence of a causative pathogen not included in the test panel should be considered and a new milk sample submitted for conventional culturing. For example, a cow with severe signs, but with a milk sample result positive only for *C. bovis* with a low amount of DNA, should automatically raise a question in the clinician’s mind as to whether the cause of mastitis might be some other pathogen not included in the test panel. Future research in PCR diagnostics should focus on defining true IMI. This could be done by testing and monitoring clinically healthy quarters, as well as those with subclinical or clinical mastitis, with PCR assays and comparing SCC or other indicators of inflammation with PCR results. Such studies require large datasets to obtain also pathogen-specific information. The relevance of the *blaZ* gene targeted in milk samples should also be looked into further, especially in samples containing *S. aureus* and non-aureus staphylococci.

7 Conclusions

This research aimed at improving the interpretation of multiplex real-time PCR results obtained from bovine mastitis cases. Conclusions based on this thesis are:

- Multiplex real-time PCR assay is a reliable tool for diagnosing *S. aureus* IMI from aseptically-taken quarter milk samples when compared with conventional culture.
- Microbes not related to IMI appear in microbiological results for milk samples tested with PCR despite using an aseptic sampling method. Environmental and minor pathogens detected with low amounts of DNA can be considered to originate mostly from extramammary sites.
- Half of the *Staphylococcus* spp., i.e. non-aureus staphylococci, in the milk samples may originate from the teat canal, teat skin or teat orifice. In PCR these are usually present with high Ct values (>34.0), indicating low numbers of bacteria. In case of *Staphylococcus* spp. (NAS), results with low amounts of detected DNA should be evaluated with high criticism.
- Detection of more than two target species, especially bacteria of environmental origin and with small amounts of detected DNA, raises concerns about the hygienic quality of the sample. In cases where the predominant bacteria cannot clearly be identified, or the sample includes more than two species, the sample should be considered contaminated.
- Bacteriological cure appears to occur faster if the sample is examined with BC compared with PCR. These samples include intact target DNA from viable, damaged or dead bacterial cells. Thus, sampling during the acute phase of mastitis increases the probability of the PCR result originating from viable cells. Based on this study we recommend waiting at least two to three weeks after the onset of mastitis or possible antimicrobial treatment, or until the quarter milk SCC has decreased to a normal level, before taking milk samples for PCR analysis to assess a bacterial cure.
- Major pathogens like *S. aureus* are present in samples from suspected IMI cases with lower average Ct values than minor pathogens like *Staphylococcus* spp. We recommend that actual Ct values would be presented in PCR analysis results instead of current estimates of high, moderate and low levels of DNA because these estimates may differ among target species.
- Mastitis diagnosis, especially when using PCR, should include all available information on the history and clinical signs of the cow, palpatory changes in the udder, changes in the milk appearance, and indicators of inflammation, including milk SCC. This information should be combined with the microbiological diagnosis. In atypical cases, the possibility of contamination or presence of a causative pathogen not included in the test panel should be considered and a new milk sample submitted for conventional culturing.

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