Intramammary infection in heifers – the application of infrared thermography as an early diagnostic tool

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Submitted in fulfilment of the requirements for the
Degree of Masters of Veterinary Medicine

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September 2018

Abstract

Mastitis is mainly caused by intramammary infection (IMI) with bacteria. Heifer IMI in early lactation impacts negatively on welfare, milk production and longevity in the herd. Prevention of subclinical and clinical mastitis caused by IMI with major pathogens, such as *Streptococcus uberis* and *Staphylococcus aureus*, could be improved if more information about the origin of heifer IMI were available. The challenges of establishing when in the pre- or peri-partum period the infection occurs make targeting of preventive management difficult. It usually requires the collection of mammary secretion prior to calving through invasive techniques, which can in itself contribute to an increased risk of infection. Non-invasive techniques, such as infrared thermography (IRT) have been applied and validated as a method for diagnosing mastitis in adult cows in controlled environmental conditions, but not in heifers under field conditions. Therefore, this study aimed to evaluate the usefulness of IRT for early detection of IMI in primigravid dairy heifers in the last trimester of gestation. The study was conducted on a commercial dairy farm with approximately 700 lactating cows. One hundred and twenty Holstein-Friesian heifers in the last trimester of gestation were enrolled in the study. IRT images of the udders were collected before calving (the first set two months prior to estimated calving date and the second set two weeks prior to estimated calving date), using a FLIR E300 infrared camera. Each set of IRT images included two projections of the udder, one caudocranial and one ventrodorsal, whereby the caudocranial projection is commonly used in lactating cow studies but the ventrodorsal projection data is easier to collect. IRT images were analysed using the geometric analysis tool of ThermaCAM Research 2.10 Pro software to obtain descriptive parameters. At calving, colostrum samples were collected aseptically from individual quarters for standard aerobic bacteriological culture and somatic cell count measurement by flow cytometry. IMI was defined as the presence of ≥10 colony-forming unit (cfu) per culture plate. Udder temperature gradients were used for the statistical analysis. Synchronization of IRT image collection with farm routines allowed for easy, fast and non-invasive collection of udder surface temperature data. IMI prevalence measured by bacterial culture of colostrum from all quarters was 20% with the majority of bacteria isolated belonging to the group of coagulase negative staphylococci (CNS; 89.7%). SCC geometric means at

calving were 336, 365 and 867 (x1000 cells/ml) for mammary quarters that were culture-negative, culture-positive with <10 cfu/plate and IMI positive, respectively. Ventrodorsal projection of the udder could replace caudocranial projection for assessment of udder surface temperature, based on the strong correlation between temperature data from both projections (e.g. r = 0.91 and r = 0.97 for $dT_{,max,}$ two months prior to calving and 2 weeks prior to calving, respectively). In addition, the ventrodorsal projection allowed for the detection of a lower maximum temperature in quarters with likely worst outcome (IMI, major pathogen and higher SCC). Nevertheless, IRT was unreliable as predictor of IMI with minor pathogens or elevated SCC (p > 0.05; binomial logistic regression and ordinal logistic regression, respectively). The method had very limited accuracy for discrimination between infected and not infected quarters (area under the receiver operating characteristic curve close to 0.5). Thus, although the collection of IRT data is a practical and non-invasive method that can easily be incorporated into the heifer management routine on a large dairy farm, it cannot be recommended as a screening tool for early and non-invasive detection of heifer IMI before calving.

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Acknowledgement

This study would not have been possible without the scholarship funding provided by the School of Veterinary Medicine – University of Glasgow, the firm of Robert Neill and Barr Farms Ltd., or without the collaboration and help of several people to whom I would like to express my very profound gratitude.

First and foremost, I thank Professor Ruth Zadoks for supervising my Masters project and her immeasurable contribution. Her support, guidance and constructive suggestions were essential to steer me in the right direction and for the conclusion of this thesis. I was very fortunate for the opportunity to learn from her throughout the project.

Mr. Timothy Geraghty for starting this project from scratch with me. His and Mr. Lorenzo Viora's supervision and encouragement during the project were very important.

The farmer for the permission to run the study on his farm, access to farm and individual animal data, and collaboration of the farm staff.

Herd manager and Miss Mairead Campbell for their contribution to the study with milk sampling collection.

Dr. Dominic McCafferty for his enormous contribution to the study providing the infrared camera, and advice on the methodology, image and statistical analysis.

Dr. Libby Graham for the possibility to work in the microbiology laboratory. Mr. Manuel Fuentes and Mrs. Kathleen Reynolds for the practical support during the bacteriological work and help with logistics of bacterial culture materials.

Dr. James Gibbons from University College Dublin for the collaboration and species confirmation of bacterial isolates.

Finally, to my family and friends for the unfailing support and continuous encouragement.

Author's Declaration

I, Patrícia Belinda Alves Simões, declare that the work in this thesis is original, and was carried out solely by myself or with due acknowledgements. It has not been submitted in any form for another degree or professional qualification.

Patrícia Simões

Parts of this thesis have been accepted for presentation and publication elsewhere:

P.B.A. Simões, T.E. Geragthy, K. Reynolds, L. Viora, D.J. McCafferty, R.N. Zadoks. Evaluation of thermography as an udder screening technique in pre-calving dairy heifers - Preliminary results. TotalDairy2015, June 2015.

P.B.A. Simões, T.E. Geragthy, L. Viora, D.J. McCafferty, R.N. Zadoks. Use of infrared thermography to diagnose mastitis in pre-partum dairy heifers. Abstract published in the World Buiatrics Congress 2016 Book of Abstracts, July 2016.

P.B.A. Simões, M. Campbell, L. Viora, J. Gibbons, T.E. Geraghty, P.D. Eckersall, R. Zadoks. Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving. Research in Veterinary Science (2017). doi: 10.1016/ j.rvsc.2017.05.024

P.B.A. Simões, M. Campbell, L. Viora, J. Gibbons, T.E. Geraghty, P.D. Eckersall, R. Zadoks. Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving. Abstract published in the Proceedings of the Fifth DairyCare Conference 2018, March 2018.

P.B.A. Simões, L. Viora, T.E. Geragthy, J. Gibbons, D.J. McCafferty, R.N. Zadoks. Use of infrared thermography in pre-partum dairy heifers to predict elevated SCC and intramammary infection at calving. Abstract published in the World Buiatrics Congress 2018 Book of Abstracts, August 2018.

Abbreviations

‘avg’	Average temperature
APPs	Acute phase proteins
AUROC	Area under receiver operating characteristic curve
BHI	Brain heart infusion broth
BVD	Bovine viral diarrhoea
Cd-Cr	Caudocranial
CFU	Colony-forming unit
cm	Centimetre
CM	Clinical mastitis
CMT	California mastitis test
CNS	Coagulase-negative staphylococci
DCC	Differential cell count
df	Degrees of freedom
DHI	Dairy herd improvement
DIM	Days in milk
DNA	Deoxyribonucleic acid
dT	Gradient temperature
$dT_{'max'}$	Maximum gradient temperature
$dT_{'min'}$	Minimum gradient temperature
$dT_{'spot'}$	Spot gradient temperature
EC	Electrical conductivity
ELISA	Enzyme-linked immunosorbent assay
ε	Emissivity
Fig	Figure
FMD	Foot-and-mouth disease
H	Kruskal-Wallis test value
Hpt	Haptoglubin
IgA	Immunoglobulin A
IgG1	Immunoglobulin G1
IgG2	Immunoglobulin G2
IgM	Immunoglobulin M
IMI	Intramammary infection

IQR	Interquartile range
IRR	Incidence risk ratio
IRT	Infrared thermography
LnSCC	Log-transformed SCC using natural logarithm
LnSCC1	Log-transformed SCC at first milking using natural logarithm
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
'max'	Maximum temperature
'max-min'	Difference between maximum and minimum temperature
MHC	Major histocompatibility complex
MHC II	Major histocompatibility complex class II
MHpt	Milk haptoglobin
'min'	Minimum temperature
n	Number
NEB	Negative energetic balance
NMC	National Mastitis Council
NML	National Milk Laboratory
OR	Odds ratio
p	*p*-value
PCR	Polymerase chain reaction
PD	Pregnancy diagnosis
PMN	Polymorphonuclear leukocytes
Q_1	First quartile
Q_3	Third quartile
r	Pearson's correlation coefficient
r^2	Coefficient of determination
ROC	Receiver operating characteristics curve
SAA	Serum amyloid A
SCC	Somatic cell count
SCC1	Somatic cell count at first milking
SE	Standard error
Se	Sensitivity
Sp	Specificity
T_{amb}	Ambient temperature

T_s	Surface temperature
$T_{s'max'}$	Maximum surface temperature
$T_{s'min'}$	Minimum surface temperature
$T_{s'spot'}$	Spot surface temperature
Vt-Dr	Ventrodorsal
U	Mann-Whitney test value
UK	United Kingdom
χ^2	Chi square test value
z	z-score

1 Introduction

Mastitis, inflammation of the mammary gland, is one of the most common diseases affecting dairy cattle. It is well recognized as a serious problem and a costly disease (Kossaibati and Esslemont, 1997; Halasa *et al.*, 2007), not just due to the direct effect on milk yield, composition and processing proprieties (Auldist *et al.*, 1996), but also because of the impact on animal welfare and its association with a higher culling risk (Bascom and Young, 1998; De Vliegher *et al.*, 2012).

Mastitis control programs aim to minimize the incidence of intramammary infection (IMI) (Dohoo and Leslie, 1991), which is mainly caused by bacteria. Heifers are usually the age group that has the highest genetic merit, and in many herds they make up the largest parity group (Compton *et al.*, 2007a). Even so, mastitis prevention programs have been overlooking nulliparous and recently calved heifers, focusing on prevention of multiparous animals' IMI instead. Prevention of heifer IMI is often limited to udder inspection and/or palpation shortly before or after calving (De Vliegher *et al.*, 2001). Quarter-level prevalence of IMI in heifers ranges between 29% and 75% before parturition, and 12% and 57% immediately postpartum (De Vliegher *et al.*, 2012). Thus, there is a high probability of heifers starting their lactation without a healthy udder, incapable of producing high-quality milk (De Vliegher *et al.*, 2004), and posing a threat to milk production and udder health during the first and subsequent lactations (De Vliegher *et al.*, 2012; Santman-Berends *et al.*, 2012), depending on the IMI-causing agent. Heifer IMI in early lactation has been highlighted as a problem of high economic importance and as a limiting factor for full expression of the genetic potential, which affects dairy farming profitability and sustainability (Piepers *et al.*, 2009; De Vliegher *et al.*, 2012). Paradoxically, an increased milk yield and reduced risk of clinical mastitis (CM) has been reported in heifers infected with coagulase-negative staphylococci (CNS) in early lactation (Piepers *et al.*, 2010).

An increase in somatic cell count (SCC) in heifers in early lactation has been considered the reflection of on-farm management measures (Dufour et al., 2011; De Vliegher *et al.*, 2012) and prevention of high SCC in this group of animals

should be focussed on reducing the level of infection prepartum and at parturition (Huijps *et al.*, 2009). However, prevention and IMI control in heifers can be difficult due to the uncertainty about the pathogenesis and epidemiology of heifer mastitis, as well as differences in risk factors between production system (Fox *et al.*, 1995; Myllys and Rautala, 1995; Waage *et al.*, 1998; Waage *et al.*, 2001; De Vliegher *et al.*, 2004; De Vliegher *et al.*, 2005).

Establishing the temporal origin of IMI would be crucial for prevention, management and reduction of the impact of heifer mastitis. However, that is still one of the biggest challenges faced by dairy farms. Most commonly, it requires invasive techniques to collect mammary secretum during the prepartum period, a procedure that can itself increase the risk of infection. Recently, several studies have explored the potential of new technological tools, such as infrared thermography (IRT), for detection of mastitis. IRT has been applied and validated as a method for assessing adult cow mastitis non-invasively, as described in detail in the literature review below, section 3.2.2.3 (Berry *et al.*, 2003; Colak *et al.*, 2008; Hovinen *et al.*, 2008; Polat *et al.*, 2010). However, it has not been applied to nulliparae. The trend towards decrease in herd numbers and increase in herd size and the associated increase in use of specialized heifer rearing units make udder health screening of heifers increasingly important before they enter the main herd, and IRT appears an attractive tool for such routine udder screening.

This thesis describes a study conducted, primarily, to evaluate the usefulness of thermography in field conditions as an early detection system for abnormal udder health during the last trimester of pregnancy in dairy nulliparae, and to gather information that could potentially help optimise herd management decisions and contribute to better heifer udder health.

2 Literature Review

Research in bovine mastitis has been driven by the necessity to ensure milk quality and animal welfare and to develop measures for the control of IMI. Commonly caused by IMI with bacterial organisms (Ruegg, 2011), mastitis continues to be a challenge for dairy veterinarians and farmers (Ruegg, 2012). Despite being widely associated with IMI, the term mastitis does not define exclusively an inflammatory response of the mammary gland to injuries caused by microorganisms, but also to traumas, allergies, physiological and metabolic changes (Alnakip *et al.*, 2014).

2.1 Overview of bovine udder structure and function

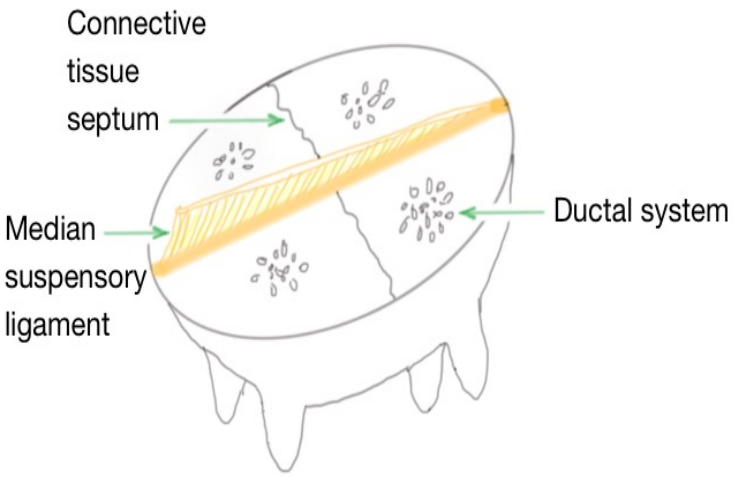
2.1.1 Udder structure

A healthy mammary gland function is extremely important for the dairy industry (Tucker, 1981) and it is related to the udder structure. The bovine udder has an inguinal location and comprises four individual secretory glands, known as quarters (Franz *et al.*, 2009). It is divided in two halves by distinct dense connective tissue, the median suspensory ligament, which is responsible for most of the strength that maintains the udder attached to the ventral abdomen (Fig 2-1A). Other ligaments involved in the udder suspensory system are the lateral ligaments. These are continuous with the median ligament and have fibres that spread over either side of the udder (Fig 2-1B) (Nickerson and Akers, 2011). Each half, left and right, has independent blood and nerve supply, lymphatic drainage and suspensory apparatus (Frandson *et al.*, 2009; Reece, 2009; Nickerson and Akers, 2011).

A division between front and back quarters within each half also exists. It is formed by a connective tissue septum, which is much less distinct than the division between halves (Fubini, 2004; Nickerson and Akers, 2011) (Fig 2-1A). Each quarter has its own milk secretory tissue (glandular parenchyma) composed of alveoli, connective tissue and ducts. Alveoli are organized in groups of 150 - 200 surrounded by connective tissue, forming the lobule. Individually, the alveolus is a small sac-like structures, arranged as a single cell layer on a basal

membrane, and surrounded by a network of smooth muscle cells, myoepithelial cells, involved in the oxytocin-induced milk ejection (Franz *et al.*, 2009; Sjaastad *et al.*, 2010; Nickerson and Akers, 2011).

A



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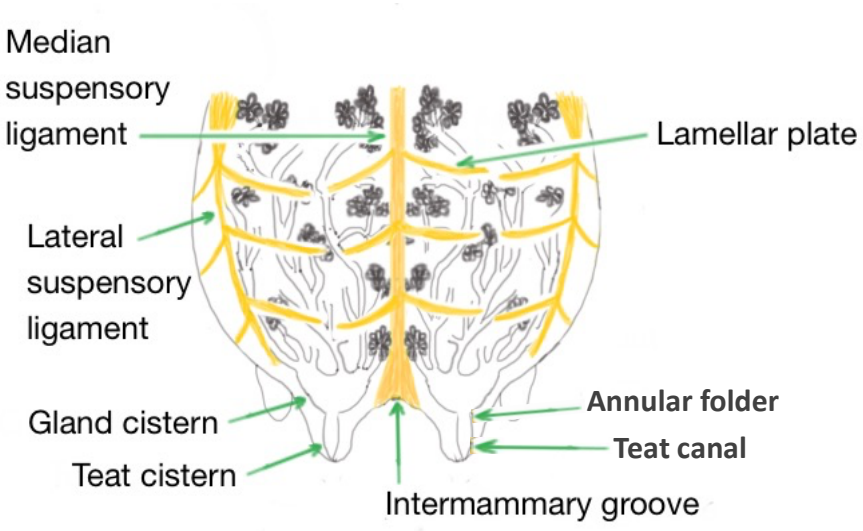

Figure 2-1 Horizontal (A) and transversal (B) cross-sectional views of bovine udder gross anatomy (based on Nickerson and Akers, 2011).

The ducts draining the alveoli converge into larger ducts that drain into a collecting space called gland cistern. This space is ventrally separated from the teat cistern by a constriction known as annular fold. The latter is smaller than the former and terminates distally at the teat canal that is surrounded by

bundles of smooth muscle fibres (sphincter) and opens at the teat orifice or meatus, through which milk is removed. At the junction of the teat cistern and teat canal is the Fürstenberg's rosette, formed by tissue folds (Paulrud, 2005; Nickerson and Akers, 2011). Approximately 60 % of the secretions synthetized are stored in the lumen or interior of the alveoli and small ducts, and 40% in the large ducts and cisterns between milkings (Nickerson and Akers, 2011).

2.1.2 Mammary gland growth until lactation

According to Tucker (1981), milk production is affected by the number of mammary cells available. The mammary gland growth starts at birth and continues until the first oestrous cycle (onset of puberty) at the same rate as the rest of the body. However, this pattern changes around puberty, with the mammary ducts and fat pad undergoing an allometric growth, undertaking a faster growth rate than the rest of the body, for a short period of time (Tucker, 1987; Squires, 2003).

Between puberty and pregnancy the mammary growth is very limited (Squires, 2003). However, conception is a crucial moment to the udder development as milk secretory gland. At this point the mammary gland starts developing alveoli (Tucker, 1987), structures responsible for the milk synthesis (Squires, 2003), which after day 150 of gestation group and form the lobule-alveolar structure (Fig 2-2). At this stage the growth rate is exponential, with the majority of the growth occurring in the last trimester of pregnancy (Squires, 2003). This phase of the gland development is stimulated by a hormonal sequence, which includes oestrogens and progesterone (ovarian and foetoplacental), prolactin (antepituitary) and corticoids (adrenal) (Convey, 1974; Delouis, 1978; Reece, 2009), and determines the number of milk-secreting cells (Squires, 2003).

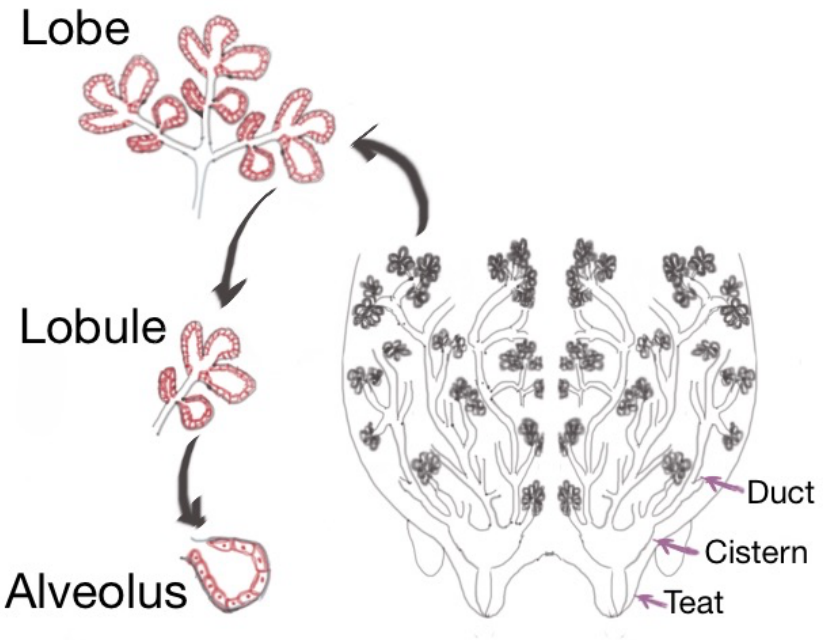

Figure 2-2 Alveolar structure of the mammary gland (based on Squires, 2003).

This hormonal sequence also has a role in the regulation of lactogenesis and may also affect the complex onset of the colostrogenesis (Barrington *et al.*, 2001).

Colostrogenesis begins three to four weeks before parturition and ceases abruptly immediately prior to parturition (Brandon *et al.*, 1971; Godden, 2008), initially with limited mammary secretion and later with a copious secretion that starts to form around zero to four days before parturition (Tucker, 1981). Colostrum, i.e. the secretion that is formed before the onset of milk production, can be found up to three to five days post parturition (Barrington *et al.*, 2001; Conte and Scarantino, 2013). It is a mixture of lacteal secretion and constituents of blood serum, which accumulate in the mammary gland during the prepartum period. The concentration of many of these constituents is greatest at the first milking and then declines over the next six milkings (Foley and Otterby, 1978; Godden, 2008).

2.1.3 Udder defences

The mammary gland is protected from penetration of microorganisms and establishment of infection by a variety of defence mechanisms, which includes anatomical, cellular and soluble (humoral) protective factors. These mechanisms

can be divided, for simplification, in innate (nonspecific or natural) and acquired (specific or adaptive), which interact in a highly coordinated manner in the modulation of the mammary gland resistance and susceptibility to infection (Sordillo *et al.*, 1997; Oviedo-Boyso *et al.*, 2007; Alnakip *et al.*, 2014).

Innate immunity is predominant in the early stages of the infection (Sordillo and Streicher, 2002; Alnakip *et al.*, 2014). Its responses are present or activated quickly at the site of infection after stimuli, however they are not enhanced by repeated exposure to the same stimuli (Sordillo *et al.*, 1997; Sordillo, 2005). This type of immunity includes the first line of defence, comprising anatomical and physical barriers to the invading microorganisms at the point of entry, such as the teat canal (Sordillo *et al.*, 1997; Sordillo, 2005; Rainard and Riollet, 2006; Alnakip *et al.*, 2014), and some cellular and soluble defences, such as macrophages, neutrophils, natural killer cells and cytokines (Oviedo-Boyso *et al.*, 2007).

Effective functioning of the teat sphincter muscle around the teat canal is important to keep the teat orifice closed and to separate the interior of the mammary gland from the exterior (Oviedo-Boyso *et al.*, 2007). Keratin inside the teat canal is a waxy material that is derived from stratified squamous epithelium (Sordillo and Streicher, 2002) that lines the teat canal and, during the non-lactating period, can completely occlude it by forming a keratin plug. It provides a physical barrier that traps and hinders the migration of microorganism, thus limiting their access to the teat cistern (Miller *et al.*, 1992; Sordillo and Streicher, 2002; Rainard and Riollet, 2006; Alnakip *et al.*, 2014). Moreover, it has associated esterified and non-esterified fatty acids (lauric, myristic, palmitoleic and linoleic) with bacteriostatic function, and cationic proteins, which can bind to mastitis-causing microorganisms, increasing their susceptibility to osmolarity changes (Miller *et al.*, 1992; Paulrud, 2005; Oviedo-Boyso *et al.*, 2007; Alnakip *et al.*, 2014).

If innate immunity acts adequately, most microorganisms are prevented from entering or eliminated shortly after entrance in the mammary gland without visible milk changes or production effects (Sordillo and Streicher, 2002). If innate immunity fails, an acquired immunity response is stimulated (Oviedo-

Boyso *et al.*, 2007). In this defence mechanism, responses are slower and mediated by antibody molecules, neutrophils, macrophages and several lymphoid populations, allowing the recognition of specific determinants of a microorganism (antigen). Consequently a faster, considerably stronger, longer lasting and often more effective response will occur in the eventuality of a second exposure to the same antigen, as a result of immunological memory (Sordillo *et al.*, 1997; Sordillo and Streicher, 2002; Sordillo, 2005; Oviedo-Boyso *et al.*, 2007; Alnakip *et al.*, 2014). Major histocompatibility complex (MHC) is essential for the acquired immune response. It is a set of membrane-bound proteins expressed on the surface of certain cells, such as B cells and macrophages, that can combine with antigens and have an important role in antigen presentation (Sordillo and Streicher, 2002).

Briefly, when a microorganism (pathogen) successfully enters the mammary gland through the teat orifice, penetrates the teat canal, evades the cellular and humoral defences, and invades and multiplies inside the gland, IMI occurs. (Sordillo, 2005; Oviedo-Boyso *et al.*, 2007). As a result, the immune response is activated and the number of somatic cells (SCC, including polymorphonuclear neutrophils (PMN), macrophages, lymphocytes and epithelial cells) increases shortly after the recognition of the antigen (Harmon, 1994; Oviedo-Boyso *et al.*, 2007; Baumert *et al.*, 2009). This host response is known as inflammation of the mammary gland or mastitis. The magnitude and onset of this SCC increase depends on the IMI causing-microorganism and the speed of neutrophil recruitment from the bloodstream, and varies with the severity of the mastitis (Rainard and Riollet, 2006; Oviedo-Boyso *et al.*, 2007).

2.2 Overview of diagnostic tools

The expansion of dairy herds and rising production levels of individual animals have increased the demand for rapid, reliable and affordable diagnostic methods that allow for early detection of mastitis and IMI and early intervention to minimise their spread and impact in the herd. The term IMI refers to the presence of an infectious organism in the mammary gland (Berry and Meaney, 2006). IMI is the most common cause of mastitis, which can manifest as subclinical mastitis (inflammation of the udder that does not cause obvious

changes to the milk aspect, mammary gland or animal health, but that can be detected by a rise in SCC or other indicators of inflammation), CM (evident signs of inflammation in the udder, changes in the milk appearance and possibly systemic signs) or non-functional or "blind" quarters (De Vliegher *et al.*, 2012). Mastitis (inflammation) does not necessarily imply infection (Dohoo *et al.*, 2011) and IMI is not necessarily associated with inflammation (Duarte *et al.*, 2015), i.e. in the case of latent infection (Fig 2-3). However, IMI and mastitis have been used almost interchangeably in the scientific and veterinary literature (Barkema *et al.*, 1997; Deluyker *et al.*, 2005).

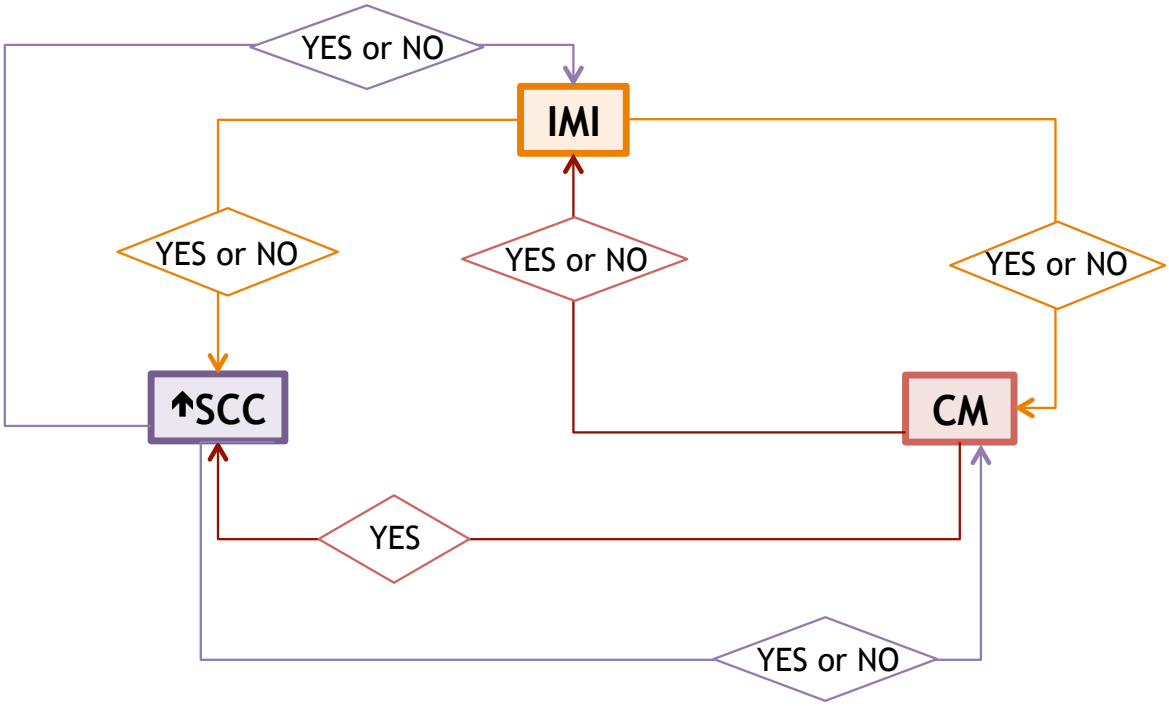

Figure 2-3 Relationship between intramammary infection (IMI), somatic cell count (SCC) and clinical mastitis.

There are several methods for detection of elevated SCC (characteristic of udder inflammation), IMI and CM available for the dairy industry (Pyörälä, 2003; Viguier *et al.*, 2009; Deb *et al.*, 2013; Duarte *et al.*, 2015), and the main methods are described here.

2.2.1 Detection of somatic cells

Somatic cell count is widely used as standard method to identify animals with subclinical mastitis (Barkema *et al.*, 1999; Åkerstedt *et al.*, 2006) and it

continues to be used as parameter in the European raw milk quality programs (EC 853/2004). It is considered a useful indicator of the average udder health (inflammation) and IMI status in the herd (Barkema *et al.*, 1997; Åkerstedt *et al.*, 2008), based on the fact that IMI is the major factor affecting SCC (Harmon, 1994; Baumert *et al.*, 2009).

In the healthy mammary gland, the predominant cell types are macrophages (34 to 79%), lymphocytes (16 to 28%), PMN (3 to 26 %) and epithelial cells (2 to 15%) (Detilleux, 2009). However, those proportions change during mastitis, with the increased milk SCC reflecting mainly the enhanced recruitment of PMN to the udder and milk as a result of chemotactic agents released during an inflammatory reaction (Lakic *et al.*, 2009). Elevated SCC can detected by semi-quantitative methods, such as California Mastitis Test, which can be performed on-farm, and by quantitative measurement of SCC, which can be performed on-farm (e.g. with portable automated cell counters) or off-farm (e.g. SCC measurement with automatic counters in laboratory settings) on quarter milk samples or on composite milk samples, i.e. samples containing milk from all functional quarters of an animal.

2.2.1.1 California Mastitis Test

The California mastitis test (CMT) is an easy, simple, inexpensive and rapid "cow-side" test that allows for estimation of SCC on-farm. This test has been used by farmers and veterinarians as routine test to identify inflammation in early stages and as an aid to stop the propagation of the disease in the herd (Bogni *et al.*, 2011; Deb *et al.*, 2013).

The addition of a reagent (bromocresol-purple-containing detergent) to a milk sample results in lysis of cells, release of nucleic acids and other constituents, leading to the formation of a 'gel-like' matrix consistency with a viscosity proportional to the leukocyte number or SCC (Rice, 1981; Viguier *et al.*, 2009). This reaction only occurs to a visible level when milk cell count is above 300,000 cells/ml (Rice, 1981).

Interpretation of the CMT is subjective, resulting in false positives or negatives, and hence is low sensitivity and specificity (Rice, 1981; Viguier *et al.*, 2009).

Positive results can also occur with fresh (less than 10 DIM) or nearly dry animals with high SCC in the absence of infection and inflammation, and negative results may occur in some cases of acute CM, when leukocytes are destroyed by bacterial toxins (Rice, 1981; Safi *et al.*, 2009).

2.2.1.2 Somatic cell count measurements

There are several portable cell counters available in the market for on-farm SCC measurement. They operate optically by staining nuclear DNA with propidium iodine (e.g. DeLaval cell counter, DeLaval International AB, Tumba, Sweden) or based on an esterase-catalyzed enzymatic reaction (e.g. Portacheck somatic cell counter, Portacheck Inc., Moorestown, NJ) (Duarte *et al.*, 2015). These are cost-effective, easily operated and rapid. However, their sensitivity at low SCC has been considered poor (Viguier *et al.*, 2009).

SCC measurements for dairy herd improvement programmes (DHI) are generally done in a laboratory and operate on the principle of optical fluorescence, e.g. Fossomatic, Foss, Hillerød, Denmark. It uses the fluorescent signal generated by the penetration and intercalation of ethidium bromide with nuclear DNA to estimate the SCC in milk. It is a rapid and automated method, however it requires an expensive and complex to use device (Viguier *et al.*, 2009).

Indicative thresholds of SCC have been used for detection of subclinical mastitis in composite milk samples, with differentiation between primiparous and multiparous animals. In Europe, a threshold of ≥200,000 cells/ml is often used for the multiparae (Schukken *et al.*, 2003; Madouasse *et al.*, 2012), and a much lower threshold for primiparae, ≥100,000 cells/ml (Harmon, 1994; Bludau *et al.*, 2014). However, the geometric average SCC is under 100,000 cells/ml for animals without IMI, including multiparous animals (Schukken *et al.*, 2003; Sampimon *et al.*, 2009).

The use of thresholds made it easier to interpret SCC results, however different thresholds represent different sensitivity (*Se*) and specificity (*Sp*) of the test. For example, a threshold of 200,000 cells/ml in composite milk has been reported with 70% of *Se* and *Sp* for the likely presence of an IMI in at least one quarter (Dohoo and Leslie, 1991; Schepers *et al.*, 1997; Green *et al.*, 2008). However,

the use of composite milk may lead to undetected infected quarters when for example just one quarter has a moderate SCC increase (Grönlund *et al.*, 2003). Furthermore, SCC does not always correlate with the infectious status of the mammary gland, and it can be affected by several physiological factors, such as production level, breed, season, lactation number and stage of lactation (Schepers *et al.*, 1997; Lakic *et al.*, 2009). For example, it is frequently increased around parturition, which is partly attributed, to physiological changes (Barkema *et al.*, 1999; Bludau *et al.*, 2014).

In relation to heifers, SCC has been used to define when IMI reached the level of herd problem rather than just an individual animal problem. A herd was considered to have a mastitis problem when more than 15% of heifers presented with a first test-day SCC (between 10 and 35 DIM) higher than 150,000 cells/ml or more than 15% of heifers were diagnosed with CM around parturition (De Vliegher *et al.*, 2012). SCC analysis has been attempted for mammary gland secretions from breeding age and pregnant heifer to detect early inflammation and study potential reductions in future milk yield, but the volume of secretion was very low and SCC was high, even in uninfected quarters, indicating practical constraints and lack of diagnostic specificity (Nickerson, 2009).

2.2.1.3 Differential cell count

Differential cell count (DCC) is a direct method that can be used to identify if the mammary gland is healthy or inflamed, with a *Se* of 97.3% and *Sp* of 92.3% even at low SCC (from 1,000cells/ml) (Pilla *et al.*, 2013). Changes in relative cell proportions in quarter milk samples are detected by cytometry, using a logarithmic polymorphonuclear neutrophilic leukocyte:lymphocyte ratio (Pilla *et al.*, 2012).

2.2.2 Detection of intramammary infection

The most common diagnostic method used for IMI detection varies geographically. For example, in the UK microbiological culture continues to be the preferred method, while in the Nordic countries polymerase chain reaction (PCR) based assays have largely replaced conventional microbiological culture.

2.2.2.1 Microbiological culture

The identification of pathogens causing IMI is important for disease control strategy and epidemiological studies (Kalmus *et al.*, 2006; Bogni *et al.*, 2011). Microbiological culture of milk samples obtained from suspect individual quarter (quarter level) or composite (cow level) samples has been used as standard diagnostic method for lactating animals (Ruegg, 2011). For identification of IMI in nulliparae it is necessary to collect prepartum mammary secretion samples, which is unpopular because it requires the opening of the teat end or other invasive techniques (e.g. needle aspiration), creating the possibility of inadvertent introduction of infection during or after sampling, especially when strict hygienic conditions are not applied (Ruegg, 2011). Conventional identification of bacterial species is generally performed in an off-farm laboratory by culture, biochemical and serological tests (Bogni *et al.*, 2011), and it is still considered the gold standard for IMI detection (Viguier *et al.*, 2009; Madouasse *et al.*, 2012). However, it has several disadvantages, i.e. it is time-consuming and labour-intensive, it requires specific media and training, the waiting time for results can be days and results may be available too late to inform clinical decision making on treatment of CM (Viguier *et al.*, 2009; Tan *et al.*, 2012; Deb *et al.*, 2013). Microbiological culture is also insufficient for identification of CNS to species level (Bes *et al.*, 2000; Capurro *et al.*, 2009; Sampimon *et al.*, 2009; Piessens *et al.*, 2011), requiring follow up with molecular techniques for accurate species identification (Zadoks and Watts, 2009).

Several on-farm bacteriological culture systems are increasingly used on North American dairy farms (e.g. Minnesota Easy Culture System (University of Minnesota, St. Paul), Accumast (FERA Animal Health LCC, Ithaca, New York) and AC Petrifilm (3M Canada, London, Ontario) and they have become available in Europe in the last few years (e.g. VetoRapid, Vétoquinol, France). Their main benefit is the possibility to be used and interpreted by farmers with limited training in microbiology and the provision of results within 24 hours, which can be useful to guide the strategic selective treatment of CM (Lago *et al.*, 2011; Ganda *et al.*, 2016) and dry cow therapy (Cameron *et al.*, 2013), allowing the reduction of antimicrobial use and amount of discarded milk (Lago *et al.*, 2011).

Culture, like SCC measurement, has imperfect sensitivity and specificity, e.g. due to variability in sample collection methods and shedding patterns of mastitis pathogens (Sears *et al.*, 1990; Ruegg, 2011).

2.2.2.2 MALDI-TOF MS

Mass spectroscopy using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) is a reliable, easy to use and cost-effective technique that can identify bacterial species and strains in few minutes, using small quantities of milk (Barreiro *et al.*, 2010; Bizzini and Greub, 2010; Raemy *et al.*, 2013; Duarte *et al.*, 2015). However, it is limited by the spectrum of bacterial protein profiles in the database available (Bizzini and Greub, 2010). In addition, it requires culture of bacteria prior to MALDI-TOF MS based identification, unless the concentration of bacteria in the milk sample is high (Barreiro *et al.*, 2017).

2.2.2.3 Polymerase chain reaction

Among the nucleic acid-based testing methods, polymerase chain reaction is one of the most popular to directly detect nucleic acids from infectious agents (Malou and Raoult, 2011). Several multiplex and 'real-time' PCR assays can simultaneously detect different mastitis-causing microorganisms in milk samples, including eighteen of the major bacterial species or genera such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis* (Koskinen *et al.*, 2009; Viguier *et al.*, 2009). Both viable and nonviable microorganisms can be detected, as well as viable but non-culturable bacteria. These methods are highly sensitive and specific, can be performed rapidly (within few hours) and overcome the sensitivity and time-constraints encountered with culture-based methods (Studer *et al.*, 2008; Viguier *et al.*, 2009; Amin *et al.*, 2011; Deb *et al.*, 2013).

However PCR diagnostic methods present drawbacks, including the detection of a limited number of bacterial species and no detection of non-bacterial species such as *Prototheca* or yeast (Yamagishi *et al.*, 2007; Deb *et al.*, 2013). In addition, contaminated samples may give false positive results, whilst the detection of dead microorganism does not give indications for use of antimicrobial treatment. Failure to differentiate between dead bacteria, which would not require treatment and live microorganisms, which may be an

indication for antimicrobial therapy, may not be appropriate in the era of prudent use of antimicrobials and the need to limit the risk of selection for antimicrobial resistance. Finally, in multiplex PCR competition can occur between different sets of primers, reducing its sensitivity (Amin *et al.*, 2011; Deb *et al.*, 2013) and 'real-time' PCR requires equipment and consumables that makes the technique difficult to afford (Deb *et al.*, 2013).

2.2.3 Detection of clinical mastitis

Clinical mastitis detection is performed by the farmer, milker, or milking machine, through visualization and palpation of the udder, and visualization or measurement of the milk appearance or temperature. Changes in blood flow and vascularity due to inflammation of the mammary gland can make the udder reddish, swollen, hard, hot and painful. Most commonly, gross changes in the milk such as changes in colour or consistency, flakes, clots, watery or serous-like milk can be detected by people during foremilk stripping of each quarter, whilst milking machines, notably automated milking systems or "milk robots" mostly detect changes in colour and temperature.

2.2.4 Additional tools

2.2.4.1 Electrical conductivity

Electrical conductivity (EC) measures the increase in milk conductivity resulting from the increase of ion levels such as sodium, potassium, calcium, magnesium and chloride during inflammation (Viguier *et al.*, 2009). EC is relatively commonly used for in-line detection of mastitis in some milking systems. However, this analysis has been reported as being less efficient in detecting chronic subclinical mastitis (Nielen *et al.*, 1995; Grönlund *et al.*, 2005), and presents diagnostic problems due to non-mastitis-related variations in milk conductivity (Viguier *et al.*, 2009). It is still considered a useful indicator, although it is not reliable or sensitive and specific enough for a conclusive diagnosis on its own (Pyörälä, 2003; Norberg, 2005; Hovinen, 2006; Viguier *et al.*, 2009).

2.2.4.2 Acute phase proteins – milk haptoglobin

Haptoglobin (Hpt) belongs to a group of blood proteins that can suffer dramatic changes in their concentration upon external or internal challenges, such as

infection, inflammation and trauma (Murata *et al.*, 2004; Åkerstedt *et al.*, 2006). It is considered a non-specific innate immune component that is involved in the restoration of homeostasis and restraint of microbial growth in the pre-acquired immunity phase (Murata *et al.*, 2004). Hpt is produced by hepatocytes, increasing under stimulation of cytokines (Murata *et al.*, 2004; Petersen *et al.*, 2004), and it is also synthetized locally in the mammary gland by milk leukocytes and epithelial cells (Eckersall *et al.*, 2001, 2006; Grönlund *et al.*, 2003; Hiss *et al.*, 2004; Grönlund *et al.*, 2005; Kalmus *et al.*, 2013). This makes it a potentially specific biomarker of the inflammatory status of the mammary gland when measured in milk samples (Eckersall *et al.*, 2001; Tan *et al.*, 2012).

Clinically healthy cows have undetectable or negligible levels of Hpt in milk (Grönlund *et al.*, 2005), whereas milk samples from high SCC quarters and infected quarters and those with CM presented significantly elevated Hpt concentrations (Eckersall *et al.*, 2001; Grönlund *et al.*, 2003; Nielsen *et al.*, 2004; Grönlund *et al.*, 2005; Åkerstedt *et al.*, 2007, 2008; Thomas *et al.*, 2015). Hpt concentrations differed between infected animals, depending on the mastitis-causing bacteria isolated (Hiss *et al.*, 2007; Pyörälä *et al.*, 2011). There is no consensus about the Hpt threshold to discriminate between normal and abnormal quarter health (Grönlund *et al.*, 2003, 2005; Hiss *et al.*, 2007; Safi *et al.*, 2009; Åkerstedt *et al.*, 2011; Pyörälä *et al.*, 2011), but prototypes of 'cow side' tests and in-line testing are currently being developed.

Irrespective of all advantages and disadvantages of the most common diagnostic methods, there is a common drawback: the fact that of all them require the use of mammary secretion or milk, meaning that they can be applied easily postpartum but not prepartum in pregnant heifers. Prepartum detection methods, used without forcing the opening of the teat canal or needle aspiration to collect intramammary secretion, would bring benefits for IMI management of heifers by reducing the risks associated with sample collection.

2.3 Overview of IMI associated bacteria

As in multiparae, the magnitude of the impact of heifer IMI is affected by individual immunity, the causative agent's virulence, time of onset of the

infection relative to parturition, cure or persistence of infection at the start of milk production and form of mastitis (De Vliegher *et al*., 2012). The same microorganisms involved in subclinical mastitis are frequently the cause of CM, and often those responsible for cases of heifer IMI are the same that are implicated in multiparous IMI (Fox, 2009). Over 135 different microorganisms have been isolated from bovine IMI (Watts, 1988), including gram-negative and gram-positive bacteria, algae and *Mycoplasma* species (Zadoks *et al*., 2011).

Traditionally, mastitis-causing bacteria have been divided in "contagious" (e.g. *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*) and "environmental" (e.g. *Escherichia coli*, *Klebsiella* spp., *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Pseudomonas* spp. and CNS), according to their primary reservoir and most likely mode of transmission or exposure (Ruegg, 2011). The term "environmental" has been used for opportunistic bacteria, considered less host adapted, that reside in the environment (Ruegg, 2012), primarily in moisture, mud and manure. It was assumed that opportunistic infection would happen through continuous or overwhelming exposure (Ruegg, 2011) of susceptible heifers or cows. The term "contagious" has been used when the udder of cows with infection serves as the primary reservoir for the bacteria (host adapted). In this case, the transmission would occur when teats of healthy cows are exposed to the microorganisms present in milk originated from infected udders (Ruegg, 2012). However, with the use of a variety of molecular techniques this classification has been shown an oversimplification (Zadoks and Schukken, 2006; Green and Bradley, 2013). Different strains of a bacterium can behave in different ways, with a mixture of environmental and contagious exposure and transmission patterns, as seen for example with *Strep. uberis*, *Staph. aureus* and even *Strep. agalactiae* (Fig 2-4) (Zadoks and Schukken, 2006). In addition, transmission via the contagious route is possible for any microorganism that can cause persistent subclinical mastitis and sheds sufficient colonies in milk to establish an infective dose, regardless of the bacterial species (Ruegg, 2012).

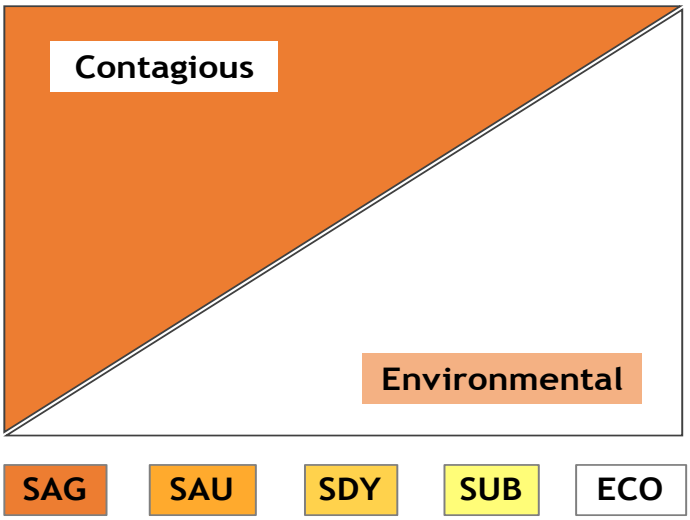

Figure 2-4 Sliding scale for contagious and environmental origin of mastitis-causing bacteria, based on insights from molecular epidemiology. Vertical axis indicates to what extent species behave as contagious (orange) or as environmental (white) bacteria. SAG – ***Strep. agalactiae***, SAU – ***Staph. aureus***, SDY – ***Strep. dysgalactiae***, SUB – ***Strep. uberis***, ECO – ***E. coli*** and ***Klebsiella*** (figure copyrighted from Zadoks and Schukken (2006), and used with author permission).

Major pathogens are bacteria considered more virulent and more prone to cause damage to the mammary gland than bacteria classified as minor (Reyher *et al.*, 2012). The former often cause CM episodes and some bacteria classified as major pathogen may persist in the udder for an extended period of time (Reyher *et al.*, 2012), as for example *Staph. aureus*. They also provoke the greatest changes in milk composition, including increases in SCC, and have the most economic impact of all causative microorganisms (Harmon, 1994). Minor pathogens usually cause much less severe reactions (Reyher *et al.*, 2012), and moderate inflammation with SCC exceeding SCC in milk from uninfected glands but not as high as in milk from glands infected with major pathogens. Minor pathogens are infrequently associated with CM, marked changes in milk composition or dramatic decreases in milk production (Harmon, 1994). *Staph. aureus*, *Strep. uberis, agalactiae* and *dysgalactiae*, *E. coli* and *Klebsiella* spp. are examples of major pathogens, whereas coagulase-negative staphylococci, micrococci, *Pseudomonas* spp., *Proteus* spp., and *Corynebacterium bovis* are examples of minor pathogens (Harmon, 1994).

The bacteria involved and their relative proportions in dairy heifers vary between herds and are affected by herd-level risk factors (Fox *et al.*, 1995;

Waage *et al.*, 1999; De Vliegher *et al.*, 2004), e.g. climate or production system, whereby pasture based systems pose different risk than systems with housing of heifers (Compton *et al.*, 2007a).

2.3.1.1 Coagulase-negative Staphylococci (CNS)

CNS is a wide group of bacteria with more than fifty different species and subspecies, however just approximately twelve have been frequently found in bovine milk samples (Tenhagen *et al.*, 2006; Gillespie *et al.*, 2009; Sampimon *et al.*, 2009; Piessens *et al.*, 2011; Supré *et al.*, 2011; De Vliegher *et al.*, 2012). Bacteria from this group have been the most prevalent microorganisms isolated in heifer IMI (Barkema *et al.*, 1999; Borm *et al.*, 2006; Parker *et al.*, 2007; Pyörälä and Taponen, 2009; Sampimon *et al.*, 2009; Krömker *et al.*, 2012) and in cases of subclinical mastitis around parturition, with a median prevalence of 31.1% prepartum and 27.9% postpartum (De Vliegher *et al.*, 2005; Fox, 2009; De Vliegher *et al.*, 2012).

Their virulence, ecology, epidemiological behaviour and effect on udder health vary between species (De Visscher *et al.*, 2015). Almeida and Oliver (2001) have shown that some species have greater virulence characteristics such as invasion of epithelial cells compared to others, with *Staphylococcus xylosus*, *Staphylococcus hyicus* and *Staphylococcus epidermidis* showing relatively high virulence in cows. However, the relevance and epidemiology of this group of bacteria as cause of bovine mastitis continues to be the subject of debate (De Vliegher *et al.*, 2009). Some studies indicate a protective effect of CNS IMI on udder health (Nickerson and Boddie, 1994; White *et al.*, 2001; Piepers *et al.*, 2010), reporting that heifers with CNS IMI were less likely to develop CM during first lactation (Piepers *et al.*, 2010; De Vliegher *et al.*, 2012) and had higher milk production than heifers without any bacteria isolated in immediate postpartum samples (Piepers *et al.*, 2010). By contrast, others implicated CNS as cause of subclinical mastitis, mild CM, decreased milk quality and production (Nickerson and Boddie, 1994; Taponen *et al.*, 2007; Pyörälä and Taponen, 2009; Schukken *et al.*, 2009). According to one study, the susceptibility to *Strep. agalactiae* IMI may increase as a result of alteration of the antibacterial components of keratin caused by CNS colonization of the teat canal (Nickerson and Boddie, 1994). Some studies have not found effects of CNS IMI on milk production (Kirk *et al.*, 1996;

Tomazi *et al.*, 2015). The diversity of the findings on the protective role or pathogenicity of CNS has been partially attributed to the classification of CNS as a homogeneous group instead of recognizing ecological and epidemiological differences between CNS species (Piessens *et al.*, 2011; Sampimon *et al.*, 2011; Vanderhaeghen *et al.*, 2014, 2015). Thus, recent publications discourage the use of CNS as a group (Sampimon *et al.*, 2011; Vanderhaeghen *et al.*, 2014, 2015) and highlight the necessity of accurate identification of the different species of CNS from milk to understand their pathological potential (Schukken *et al.*, 2009; Vanderhaeghen *et al.*, 2015).

In recent years, CNS have been isolated more frequently from quarters with CM, and they are therefore considered as emerging bacteria by some authors (Pyörälä and Taponen, 2009). It was hypothesised that heifers with higher genetic potential for milk production could be more susceptible to IMI caused by this group of bacteria (De Vliegher *et al.*, 2012) and that CNS may be relatively more important in heifers than in multiparous animals, due to a larger difference in SCC between infected and culture-negative heifers (Schukken *et al.*, 2009). As a result, and despite the differences of opinion over the importance and clinical relevance of the identification of each species, some researchers state that this group of bacteria can no longer be ignored, in particular in situations where major mastitis-causing bacteria are well controlled (De Vliegher *et al.*, 2009) or in herds with low bulk milk SCC and a high number of heifers, where CNS infections may make an important contribution to bulk milk SCC (Schukken *et al.*, 2009).

CNS are abundantly present in extra-mammary habitats such as skin of the teat apices (Borm *et al.*, 2006; Braem *et al.*, 2012, 2013; De Visscher *et al.*, 2015) and the bovine environment, such as sawdust and air (Piessens *et al.*, 2011; De Visscher *et al.*, 2015). *Staphylococcus chromogenes*, *Staphylococcus devriesei*, *Staphylococcus haemolyticus*, and *Staphylococcus equorum* are predominant species colonizing teat apices of prepartum heifers (De Visscher *et al.*, 2015), whereas *Staphylococcus chromogenes*, *Staphylococcus xylosus* and *Staphylococcus simulans* are the predominant species found in cases of IMI during lactation (De Visscher *et al.*, 2015). Although those CNS species are more common as cause of IMI in heifers than in multiparous cows (Taponen *et al.*,

2007; Sampimon *et al.*, 2009; De Visscher *et al.*, 2015), they have been associated with a longer and more pronounced increase in SCC in heifers than other CNS species (Supré *et al.*, 2011; Fry *et al.*, 2014). This suggests that these species have a more substantial effect on udder health and are, for that reason, "more relevant" than others as cause of IM (Supré *et al.*, 2011; De Visscher *et al.*, 2015). In addition, they have the ability to cause persisting infection (Piepers *et al.*, 2009; Ruegg, 2011) and cause greater increases of SCC than other species (Laevens *et al.*, 1997; Supré *et al.*, 2011; Fry *et al.*, 2014), as well as greater production losses (De Vliegher *et al.*, 2003). Of the three species most commonly found in heifer IMI, *Staph. chromogenes* is most likely to cause persistent infection (Supré *et al.*, 2011). This species of CNS has rarely been isolated from the bovine environment (Piessens *et al.*, 2011), but it has been the most common CNS species isolated in prepartum heifer secretions and primiparous milk samples in many studies (Trinidad *et al.*, 1990; Aarestrup and Jensen, 1997; Rajala-Schultz *et al.*, 2004; Oliver *et al.*, 2005; Taponen *et al.*, 2006; Pyörälä and Taponen, 2009). It shows indications of a host-adapted nature (Fry *et al.*, 2014; De Visscher *et al.*, 2015a) and is suggested to be protective to a certain extent against IMI with major pathogens, such as *Staph. aureus*, *Strep. dysgalactiae* and *uberis* (De Vliegher *et al.*, 2004), when it colonizes the teat apex. This protective role of *Staph. chromogenes* is attributed to competitive exclusion mechanisms at the teat apex (De Vliegher *et al.*, 2003; De Vliegher *et al.*, 2004).

The epidemiology of the other two predominant CNS species in heifers, *Staph. xylosus* and *simulans*, is less known and requires further study. *Staph. xylosus* appears to be a versatile species based on its occurrence in both animals and their environment, whereas *Staph. simulans* has been described as causing typically "contagious" IMI, with capacity to cause opportunistic cases of CM (Vanderhaeghen *et al.*, 2015).

Despite the frequent high prevalence of CNS in heifers IMI studies, CM cases in heifers are mostly caused by major the mastitis pathogens *Staph. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, and *E. coli* (Waller *et al.*, 2009).

2.3.1.2 Staphylococcus aureus

Staphylococcus aureus is an important cause of udder infections in dairy herds (Barkema *et al.*, 2006). Generally it presents with a lower prevalence in heifer IMI than CNS, however it has been one of the bacteria most frequently isolated from heifers (Waage *et al.*, 1999; Borm *et al.*, 2006; Sampimon *et al.*, 2009; Waller *et al.*, 2009) and its relevance should not be underestimated. *Staph. aureus* is generally referred to as a bacterium with "contagious" behaviour in lactating cattle (Paradis *et al.*, 2010). In heifers around parturition, however, it is mostly environmental, which is logical as pre-partum heifers are not exposed to contagious transmission via the milking machine (Zadoks *et al.*, 2000). It has been identified as major cause of CM in heifers (Waage *et al.*, 1999; Fox, 2009; Waller *et al.*, 2009; De Vliegher *et al.*, 2012), as well as the most difficult bacterium to control. It can persist into lactation (Roberson *et al.*, 1994; Paradis *et al.*, 2010). Purchase of heifers constitutes a risk in terms of biosecurity because introduction of heifers may result in introduction of *Staph. aureus* into a herd (Barkema *et al.*, 2006; De Vliegher *et al.*, 2012), and the number of *Staph. aureus* strains present in a herd increases as heifer purchases increase (Middleton *et al.*, 2002).

The capacity to induce persistent and chronic IMI has been attributed to its ability to invade mammary epithelial cells and evade the intramammary defence mechanisms (Sears and McCarthy, 2003). The damage caused to the mammary tissue in development in nulliparae may be responsible for the reduced milk production and quality, as well as an important factor limiting heifers in their ability to reach their maximum milk production potential during first and subsequent lactations (Owens *et al.*, 2001; Ruegg, 2011; Stalder *et al.*, 2014). *Staph.* aureus can cause elevated SCC over the entire lactation (Whist *et al.*, 2009), as well as premature culling (Stalder *et al.*, 2014). Therefore, it is considered one of the most important and costly mastitis agents (Ruegg, 2011).

The sources of infection and routes of transmission of *Staph. aureus* mastitis in heifers are still poorly understood (Stalder *et al.*, 2014). *Staph. aureus* appears to be ubiquitous in the environment (Roberson *et al.*, 1994), and has been isolated from housing, bedding, water, insects, feed utensils and other farm equipment, humans and other animal species, heifers' body sites (muzzle, teats,

rectum, vagina and intramammary secretions), bovine milk and colostrum (Roberson *et al.*, 1994; Roberson *et al.*, 1998). Some authors describe it as living primarily in the mammary gland of infected cows and infecting young heifers through feeding with colostrum and non-pasteurized milk containing the bacteria, or due to contact with objects contaminated with infected milk (Roberson *et al.*, 1998; Ruegg, 2011). Calves fed with colostrum or milk containing *Staph. aureus* could possible lick their or other calves' udder and contribute to teat skin colonization, in the same way that inter-suckling between pre-weaned heifers may also contribute to transfer bacteria among animals in a group (Ruegg, 2011). However, other studies did not yield evidence for transmission of *Staph. aureus* via inter-suckling and suggested instead that flies are important in transmission (Roberson *et al.*, 1994; Roberson *et al.*, 1998). Teat canals of nulliparous heifers can become colonized with *Staph. aureus* at a very young age (Nickerson, 2009), and heifers are at 20 times higher risk *Staph. aureus* IMI at parturition after its isolation from prepartum secretions (Roberson *et al.*, 1994). Middleton *et al.* (2002) found that 47% of the strains of *Staph. aureus* detected at parturition were the same as those strains present on the udder and teat skin of heifers before calving. In their study, strains found in heifers at parturition corresponded to the strains detected from lactating cows in 71% of the cases (Middleton *et al.*, 2002), whilst heifer strains were different from the strains isolated from lactating cows in another study (Zadoks *et al.*, 2000). Biting flies can play a role in the transmission of *Staph. aureus* between infected and uninfected heifers (Nickerson *et al.*, 1995; Ruegg, 2011). Herd management may affect the spread of *Staph. aureus* during the immediate pre- or postpartum period of heifers, in particular when the same pens are used to hold heifers and sick cattle, allowing for contamination of the bedding with secretions from those sick animals, or when fresh heifers are milked with equipment that was previously used on cows that are shedding *Staph. aureus* (Ruegg, 2011). Conversely, infected heifers may represent an important source of infection for uninfected herd mates once they enter the milking process (Roberson *et al.*, 1994).

2.3.1.3 Streptococcus uberis

Streptococcus uberis has been considered to behave mainly as "environmental" organism. It has been isolated from soil, bedding material, faeces and from

several areas on the cow, such as skin and udder (Smith and Hogan, 1993; Zadoks *et al.*, 2011; De Greeff *et al.*, 2013). A large diversity of strains occurs in the environment, resulting in infection with different stains in the same herd (Zadoks *et al.*, 2003; Bogni *et al.*, 2011; Tassi *et al.*, 2013). In lactating cattle, *Strep. uberis* is increasingly recognized to be a contagious pathogen (Zadoks *et al.*, 2003; Davies *et al.*, 2016) but this route of transmission is less likely in heifers that are not participating in the milking process yet.

Heifers have been reported to have a higher incidence of "environmental" mastitis caused by *Strep. uberis* than multiparae (McDougall *et al.*, 2009), with this bacterium being reported as cause of subclinical and CM in peripartum heifers, and as the most common major pathogen isolated in the pasture-based system in New Zealand (Pankey *et al.*, 1996; Compton *et al.*, 2007a; Parker *et al.*, 2007).

2.3.1.4 Streptococcus dysgalactiae

Streptococcus dysgalactiae has often been grouped together with *Strep. uberis* as "environmental streptococci" in laboratory reports. However, they differ in many bacteriological and epidemiological characteristics. For example, *Strep. dysgalactiae* largely conforms to the description of "contagious" in nature, even if it is also found in the environment (Fox and Gay, 1993; Bogni *et al.*, 2011). In addition, it has been reported to be able to invade epithelial cells, at least *in vitro*, and it survives there for a long period without damaging the cells (Calvinho and Oliver, 1998; Tenhagen *et al.*, 2009), similar to *Strep. uberis* and some strains of *E. coli* (Fox and Gay, 1993). It has been found at higher prevalence in primiparae than in multiparae (Tenhagen *et al.*, 2009) and is strongly associated with an increased risk of CM postpartum when infection with *Strep. dysgalactiae* occurs before or shortly after parturition (Aarestrup and Jensen, 1997; Parker *et al.*, 2007, 2008).

2.3.1.5 Escherichia coli

Escherichia coli was reported in some heifer studies (Kalmus *et al.*, 2006; Compton *et al.*, 2007a). It has been classified as an opportunistic "environmental" bacterium, and IMI usually is manifested with clinical signs. However, molecular studies have shown that *E. coli* IMI in multiparous can also

occur during the non-lactating period, without clinical signs, and become persistent until early lactation (Bradley and Green, 2000). This is an interesting aspect that perhaps might occur in nulliparae.

2.3.1.6 Mycoplasma spp.

Mycoplasma spp. is recognized worldwide as a bacterium that can cause outbreaks of mastitis in adult dairy cattle. It can also cause arthritis, urogenital disorders, pneumonia and infectious keratoconjunctivitis and abortion. *Mycoplasma* sp. is commonly involved in respiratory diseases and otitis media in youngstock, and it has also been reported as causative-agent of mastitis in young cattle. For example, *Mycoplasma bovis* was reported as the cause of CM in peripubertal heifers (Fox *et al.*, 2008) and *Mycoplasma bovigenitalium* as the cause of mastitis in a 7-week-old heifer concomitantly affected by arthritis (Roy *et al.*, 2008). The transmission of this bacterium through aerosols from other cattle with respiratory infection caused by *Mycoplasma* sp. (Barkema *et al.*, 2009), by direct inoculation into the mammary gland and by dissemination from other body sites, as for example from the respiratory mucous membranes, urogenital and joints infected (Fox *et al.*, 2005), can result in *Mycoplasma* sp. mastitis. Purchasing infected heifers is often the route of introduction of *Mycoplasma* sp. in the herd (Barkema *et al.*, 2009).

2.4 Overview of heifer intramammary infection

For many years heifers were perceived as free of IMI prepartum or at parturition (Myllys and Rautala, 1995), even after Schalm's (1942) description of mastitis cases in heifers at parturition. However, this perspective started changing after Munch-Petersen's (1970) report of 22% of heifers presenting with infected quarters at the first day of lactation. Nowadays, heifer IMI has been receiving more attention and has been recognised as a distinct problem from that in multiparous animals (Compton and McDougall, 2008).

2.4.1 Occurrence and impact of heifer IMI

During the last decades, dairy heifer mastitis has been the focus of scientific research with the aim of expanding knowledge about IMI in this group of animals

and its impact on the industry. The occurrence of IMI, possibly leading to subclinical mastitis or CM, is commonly expressed by two epidemiologic measures of disease frequency in a population, prevalence and incidence. Prevalence is a measure of occurrence at a defined time point. It is the mathematical product of incidence and duration of IMI. Incidence is the rate of occurrence of new cases of a disease/infection/condition over a defined period of time, which can be measured in calendar units (e.g. per year) or in units at risk (e.g. per lactation or per 100 animal days at risk). It is affected by susceptibility and exposure.

2.4.1.1 Somatic cell count in heifers

Based on SCC, subclinical mastitis has been reported with a higher prevalence and incidence in heifers than in multiparous animals, especially early in lactation (McDougall *et al.*, 2009). Subclinical mastitis prevalence in this period varies within a range of 16% to 35% (De Vliegher *et al.*, 2001; De Vliegher *et al.*, 2004; Svensson *et al.*, 2006; Santman-Berends *et al.*, 2012; Krömker *et al.*, 2012).

Archer *et al.* (2013a) showed that high SCC in heifers between 5 and 30 DIM was negatively associated with lifetime milk yield and that it can be an important economic predictor of future productivity. Heifers with a first test-day SCC <100,000 cells/ml produce approximately 400 kg and 750 kg more during the first lactation than heifers with 100,000 to 400,000 cells/ml and >400,000 cells/ml at the first test, respectively (Coffey *et al.* 1986; De Vliegher *et al.* 2005a). However, when accounting for losses in production by SCC it is necessary to take in consideration how many days in milk the heifer is when the SCC measurement is performed. For example, different values have been reported for heifers at the beginning of lactation (10 DIM) with a SCC of 500,000 cells/ml and 1,000,000 cells/ml, which have shown losses of production of 119 kg and 155 kg, respectively, during the first 305 days of lactation, when compared with heifers with a SCC of 50,000 cells/ml at 10 DIM (De Vliegher *et al.* 2005b). Thus, estimates of milk yield losses differ considerably between studies. De Vliegher *et al.* (2005b) have also reported that an elevated SCC at 5 DIM is less consequential for milk production than an equally high SCC at 14 DIM. The impact observed has also been related to the pathogen involved in elevating the

early SCC (De Vliegher *et al.*, 2004; De Vliegher *et al.*, 2005; De Vliegher *et al.*, 2005; Piepers *et al.*, 2010).

High SCC in early lactation also poses a risk for culling. De Vliegher *et al.* (2005a) reported that heifers with elevated SCC between 5 and 14 DIM presented a culling hazard increased by 11% per unit increase in log-transformed SCC (LnSCC). Despite the occurrence of a substantial decrease of the milk SCC of these heifers during the first weeks postpartum and an excellent udder health (≤50,000 cells/ml) at the second SCC test, these heifers were still more at risk of being culled in their first lactation than heifers with low SCC at both tests (De Vliegher *et al.*, 2005). This suggests the necessity of a strategy based on prevention rather than cure of elevated SCC in early lactation (De Vliegher *et al.*, 2005). However, high yielding heifers are generally protected from culling independent of a high first SCC test result (De Vliegher *et al.*, 2005).

Herds where ≥10% of heifers have SCC ≥400,000 cells/ml between 5 and 30 DIM would benefit economically from an improvement of heifers' prepartum udder health (Archer *et al.*, 2013a). The estimated reduction of high SCC in this period of lactation could account for savings between €50 and €140 per heifer (Archer *et al.*, 2013a). This is of even greater importance when considering the high proportion of first lactation animals in herds nowadays, together with the fact that two-thirds of the economic losses in dairy production are attributed to subclinical mastitis (Seegers *et al.*, 2003; Halasa *et al.*, 2007), which is most often due to bacterial IMI (Djabri *et al.*, 2002).

To the direct economic impact of subclinical mastitis caused by the reduction of the milk production and milk price as result of the high SCC (Carlén *et al.*, 2005), must be added the inherent costs of management of high SCC animals. Therefore, a high prevalence of heifers with elevated SCC in the early postpartum period can represent large financial losses, resulting in substantial decrease of revenues for farmers (De Vliegher *et al.*, 2005; De Vliegher *et al.*, 2005).

2.4.1.2 Intramammary infection in heifers

Bacteria have been found in mammary gland secretions and teat canal keratin as early as 6 months after birth (Trinidad *et al.*, 1990). In some parts of the world IMI affects more than 90% of heifers between 12 to 24 months of age, as reported in studies conducted in Louisiana, USA (Trinidad *et al.*, 1990; Nickerson *et al.*, 1995; Nickerson, 2009). In those studies, IMI prevalence varied with the use of fly control (Nickerson *et al.*, 1995) and most infections were caused by CNS (*Staph. hyicus* and *Staph. chromogenes*), *Staph. aureus* or mixed CNS and streptococcal species.

IMI prevalence increases towards the parturition date, reaching its highest level during the last trimester of pregnancy (Fox *et al.*, 1995; Compton and McDougall, 2008). Prepartum infection prevalence is associated with the prevalence of postpartum infection (Oliver and Sordillo, 1988; Aarestrup and Jensen, 1997), which has been attributed to the persistence of IMI diagnosed during the prepartum period, not just until parturition, but also for many days or weeks after the beginning of the lactation (Oliver and Mitchell, 1983; Pankey *et al.*, 1991; Roberson *et al.*, 1994; Aarestrup and Jensen, 1997; Oliver *et al.*, 2003; De Vliegher *et al.*, 2005). However, IMI prevalence decreases once lactation starts (Fox, 2009), suggesting higher susceptibility to IMI during the peripartum period, when a more accentuated mammary gland development takes place (Fox *et al.*, 1995; Aarestrup and Jensen, 1997; Krömker *et al.*, 2012) and clearance of infection is lower because there is no milking and hence no removal of bacteria or replenishment of removed immune cells with fresh cells.

During the first lactation, the mammary gland continues to grow (Tucker, 1987), and the presence of IMI at the beginning of the lactation may result in disturbance of the ongoing mammary gland development (Oliver *et al.*, 2003; Piepers *et al.*, 2009; De Vliegher *et al.*, 2012). This is attributed to the pathogenic effect of bacterial virulence factors and damage caused by polymorphonuclear leukocytes and other inflammatory cells on the mammary parenchyma (Nickerson *et al.*, 1995; De Vliegher *et al.*, 2005b), which leads to significant reduction of the alveolar epithelium and luminal areas (Nickerson *et al.*, 1995) and an associated increase of interalveolar stroma and leucocytes in the infected tissues (Kirk, 1984; Hallberg *et al.*, 1995).

In pasture-grazed dairies in New Zealand, heifers that presented IMI with a major pathogen within the first 5 DIM had a 60% increased risk of removal from the herd during the first lactation (Compton *et al.*, 2007a). Longevity is one of the highly desirable traits in a dairy farm with considerable repercussions on farm profitability (Sewalem *et al.*, 2004). It is generally necessary that a heifer survives beyond the first lactation to break even on its rearing costs (Hennessy *et al.*, 2011; Kennedy *et al.*, 2011; Archer *et al.*, 2013b). Rearing costs are often unknown to the farmer (Heikkilä *et al.*, 2012), but can be quite substantial (€1451/heifer; Kennedy *et al.*, 2011).

2.4.1.3 Clinical mastitis

The incidence of peripartum CM is greater in heifers than in multiparous cows (Barkema *et al.*, 1998; Valde *et al.*, 2004; Compton *et al.*, 2007a; Nyman *et al.*, 2007; Parker *et al.*, 2007; Lopez-Benavides *et al.*, 2009), with more than 30% of CM cases occurring within the first two-three weeks of lactation, in contrast to 13% in multiparous cows (Barkema *et al.*, 1999b; Svensson *et al.*, 2006; Compton and McDougall, 2008).

Peripartum CM (Edinger *et al.*, 1999) and CM in the first week of lactation substantially decreases the long term production (Oliver *et al.*, 2003; De Vliegher *et al.*, 2005). Some of the first studies published on heifer mastitis reported 1% to 5% loss in milk yield over the lactation due to CM around parturition (Oltenacu and Ekesbo, 1994; Myllys and Rautala, 1995). Additionally, cases of CM within the first week of lactation have been shown to increase the risk of CM in the subsequent lactation (Edinger *et al.*, 1999), which has subsequently been attributed to the effects on the milk secretory tissue (De Vliegher *et al.*, 2012).

One case of CM in early lactation can reach an average cost of €270/heifer/herd (calculations based on Dutch/Belgian dairy production system with milk quota). This calculation includes production losses, veterinary assistance, treatments and drugs, discarded milk, labour and culling (Huijps *et al.*, 2009). However, further losses can be added by the decreased milk quality, increased risk of drug residues, capital investments, adverse effects on other diseases (Barker *et al.*,

1998; Halasa *et al.*, 2007) and the impact on udder health problems in subsequent lactations (Rupp *et al.*, 2000).

The culling risk during the first lactation increased after a CM event in early lactation (Edinger *et al.*, 1999). Waage *et al.* (2000) report that in Norway 10.9% of the heifers treated due to CM immediately prepartum or postpartum were culled within the first 28 DIM, as a consequence of the damage caused to the udder, corresponding to a 2.4 times greater risk of being culled than non-mastitic heifers.

2.4.2 Risk factors

Poor udder health in heifers is likely to be associated with numerous genetic, physiological and environmental factors that can compromise host immunity (Sordillo and Streicher, 2002) and with environmental exposure to high pathogen loads, which may increase the incidence of IMI.

In the past decades, several studies tried to clarify how dairy heifers are infected by aetiological agents of IMI, however several questions remain unanswered and the infection pathway remains unclear (Castelani *et al.*, 2013). Nevertheless, all studies have assumed that heifer IMI is caused by bacteria from the normal teat skin flora, oral and nasal cavities, respiratory secretions, waste milk and colostrum, bedding and manure, older cows and other environmental sources (Oliver and Mitchell, 1983; Fox *et al.*, 2008; Foster *et al.*, 2009; Maunsell *et al.*, 2011; Aebi *et al.*, 2012; De Vliegher *et al.*, 2012; Bludau *et al.*, 2014; Lysnyansky *et al.*, 2016).

The epidemiology and risk factors of IMI for heifers are different from multiparous cows (Parker *et al.*, 2007; McDougall *et al.*, 2009; Santman-Berends *et al.*, 2012), in part because nulliparae are not milked and are often raised remotely from the lactating herd, and therefore exposed to different environmental risk factors (Parker *et al.*, 2007). In addition, primigravid heifers are still growing and continue growing after parturition when they are undergoing adaptation to lactation, lactating housing and management, and to the milking process. IMI risk factors are farm specific, but there are suggestions

that their effects can be controlled and minimised through herd management measures (Parker *et al.*, 2007; Fox, 2009).

2.4.2.1 Climate, season and geographical location

Climate and season, intrinsically related with geographical location, have been related to the risk of elevated SCC, IMI and CM in heifers. In addition, there are variations between herds in the same geographical location, indicating that heifer management also influences IMI prevalence and plays an important role in the prevention of IMI (Fox *et al.*, 1995; De Vliegher *et al.*, 2001; Fox, 2009).

Fox *et al.* (1995) conducted a study in several states of the USA (California, Louisiana, Vermont and Washington State) and reported a significant effect of location on IMI prevalence in breeding age heifers (included nulligravid and primigravid, from 8 to 38 month-old) and following parturition. Prevalence was highest in heifers from Louisiana. This state has the hottest and most humid climate of the states in the study. Season influenced IMI prevalence following parturition in all states, with IMI being more prevalent in the summer when *Staph. aureus* and environmental bacteria were isolated more frequently (Fox *et al.*, 1995).

In the Nordic countries, parturition in late spring and summer was associated with greater risk of CM than at other times of the year (Oltenacu and Ekesbo, 1994; Waage *et al.*, 1998). However, heifers kept on pasture during the summer and autumn were at decreased risk of CM in comparison with heifers of nongrazing herds, when parturition occurred during pasture season (Waage *et al.*, 1998).

De Vliegher *et al.* (2001, 2004b) reported seasonal variation of SCC in early lactation heifers in Belgium, which has temperate climate. Heifers that calved from April to June had a higher risk of elevated SCC than heifers calving in the other months of the year (De Vliegher *et al.*, 2004b). Higher SCC was considered the result of keeping heifers confined indoors for long periods, either in free-stall barns or other types of barn, increasing the risk of getting IMI (De Vliegher *et al.*, 2004b).

2.4.2.2 Housing system/management

Heifers are frequently exposed to stressful situations, such as multiple movements between groups, pens/sheds and different housing systems, which take place around parturition. Such stressors can have a negative impact on their immune defence, contributing to an increased risk of IMI (Mallard *et al.*, 1998; Nyman *et al.*, 2009).

For example, it is not uncommon to move heifers from the heifer-rearing unit into a close-up pen, followed by the calving pen, fresh pen and finally the lactating cow accommodation. Those movements are farm specific, but have been demonstrated of importance as risk factor for IMI. For instance, the absence or short duration of a period of adaptation to new housing before parturition has been associated with an increase in clinical and subclinical mastitis at parturition and in early lactation (Kalmus *et al.*, 2006, Svensson *et al.*, 2006). Similarly, Kalmus *et al.* (2006) reported an increased risk of CM at parturition when heifers were moved too late to the calving facilities. Barkema *et al.* (1999) reported that the risk of CM increased when heifers are commingling with dry cows before parturition.

Housing conditions may also affect the risk of heifer IMI, in particular the hygiene of the calving pen. As expected, a relation was found between poor hygiene of the calving pen and greater prevalence of high SCC (Bareille *et al.*, 2000; De Vliegher *et al.*, 2004), as well as higher probability of infection shortly after parturition (Piepers *et al.*, 2011). In New Zealand, where dairy cattle are kept on pasture all year round and where *Strep. uberis* is the most common cause of heifer mastitis, poor udder hygiene postpartum had an incidence risk ratio (IRR) of 1.32 for subclinical mastitis (Compton *et al.*, 2007b).

Other accommodation aspects that have been related to a higher risk of mastitis are the type of floor and bedding in the calving pen. Non-slatted floors, as opposed to slatted, were found to increase the odds of heifers having a high SCC. This effect was associated with wet bedding (Barkema *et al.*, 1999; De Vliegher *et al.*, 2004). Mucking out the calving pen less often than once a month constituted a significant risk for CM (Peeler *et al.*, 2000). Bedding materials, such as sawdust, shavings ((Nyman *et al.*, 2009) and organic bedding material

(Krömker and Friedrich, 2009) and their way of storage were also related with poor udder health. Storing bedding materials for prepartum heifers indoors rather than outdoors could reduce the odds of high SCC within the first 30 DIM by 21% (Green *et al.*, 2008).

High stocking density and increased time spent on feeding areas have been suggested to increase environmental exposure to mastitis-causing agents and the risk of underfeeding, leading to decreased immune function and a higher proportion of heifers with CM when compared with herds that had lower stocking rates (Parker *et al.*, 2007).

2.4.2.3 Flies

Flies have been reported as an important source and risk factor of IMI in heifers, particularly for *Staph. aureus* (Nickerson *et al.*, 1995; Fox, 2009; Anderson *et al.*, 2012). Herds using fly control had lower prevalence of IMI than herds without fly control (Nickerson *et al.*, 1995). In addition, the identification of identical genotypes of *Staph. aureus* in horn flies (*Haematobia irritans*), heifer body sites (mouth, nostrils and teats) and heifer colostrum support the role of flies in IMI (Nickerson *et al.*, 1995; Fox, 2009; Anderson *et al.*, 2012). Flies may also be associated with the transmission of the summer mastitis complex. This type of mastitis is caused by *Tuerepella pyogenes* with or without *Peptostreptococcus indolicus* or *Strep. dysgalactiae* and it is relatively common in heifers and dry cows (Fox and Gay, 1993).

2.4.2.4 Feeding management

In 1942 Schalm concluded that feeding mastitic milk with *Streptococcus agalactiae* to heifer calves would result in subsequent IMI with this bacterium at first parturition, potentially as a result of cross-suckling (Schalm, 1942). However, the relation between feeding mastitic milk to calves and subsequent IMI was considered unclear by Kesler in 1981 in a review paper.

The increase of concentrate feeding to heifers between 11 and 16 months of age has been reported as a risk factor for elevated SCC in early lactation (Svensson *et al.* 2006). Selenium and vitamin E provision can lower the risk of CM (Weiss *et al.*, 1997; Barkema *et al.*, 1998), but the benefit of selenium and vitamin E

supplementation depends on whether or not there is a deficit prior to supplementation (Leblanc *et al.*, 2002; Ceballos-Marquez *et al.*, 2010). Differences between countries and regions should be considered when supplementing a diet with vitamins, because adverse effects on udder health have been reported for supplementation of vitamin E and selenium in non-deficient cows (Bouwstra *et al.*, 2010).

Suboptimal nutrition can lead to a negative energetic balance (NEB), which has been associated with a decrease in the mammary gland immunity (Suriyasathaporn *et al.*, 2000). Primiparae are still growing during early lactation, which needs to be accounted for in ration calculations (König *et al.*, 2008). Heifers with medium and high fat:protein ratios in milk, an indicator of increased tissue mobilization associated with NEB, had higher prevalence of subclinical mastitis than heifers with low fat:protein ratios (Bludau *et al.*, 2014). Elevated beta-hydroxybutyrate levels in blood, which may also result from NEB, and body condition loss were associated with greater risk of udder oedema and indirectly with greater incidence of CM, in grazing heifers (Compton *et al.*, 2007b).

2.4.2.5 Last trimester of gestation

During this period, several dramatic alterations take place and the mammary gland susceptibility to IMI increases (Sordillo and Streicher, 2002; Nyman *et al.*, 2009). This is caused in part by considerable fluid accumulation and increased intramammary pressure, and subsequent teat canal dilation and leakage of mammary secretions (Oliver and Sordillo, 1988; Sordillo, 2005). In addition, neutrophil functions are altered or impaired around calving (Kehrli *et al.*, 1989; Heyneman *et al.*, 1990; Zecconi *et al.*, 1994), due to an increase of immature neutrophils in the blood and the resulting lower number of mature neutrophils in blood and mammary secretions (Sordillo and Streicher, 2002). Mammary gland macrophages also have altered functional capabilities during this period, which is directly linked with disease incidence. Macrophages are present in higher numbers in the last week of gestation but their phagocytic capacity is decreased, possibly as a result of lower opsonic activity in mammary secretions (Sordillo and Streicher, 2002). Additionally, their MHC II expression is decreased, which may contribute to poor antigen presentation and weaker specific immune

responses from mammary gland lymphocytes (Fitzpatrick *et al.*, 1992; Mallard *et al.*, 1998).

Alterations in host defences are especially notable two weeks prepartum, but they commonly remain through the first three weeks of lactation (Sordillo *et al.*, 1997; Sordillo and Streicher, 2002).

2.4.2.6 Keratin plug

During the non-lactating period, the teat canal of multiparous cows contains a keratin "plug" that is protective against new IMI (Dingwell *et al.*, 2004). Absence of this keratin "plug" and poor closure of the teat canal may increase the risk of IMI in the last weeks of gestation (Compton *et al.*, 2007b). Up to 60% of heifers may have teat canals not sealed by keratin ("open quarters") at ten to four weeks prepartum, and may be influenced by host genetic effects (Williamson, 2002; Krömker and Friedrich, 2009). It was not proven that quarters with a relatively long time ''open'' suffered an increased risk of becoming mastitic after parturition compared to those that were open for a short time only (Krömker and Friedrich, 2009).

2.4.2.7 Udder oedema

Udder oedema is characterized by an accumulation of interstitial fluid in the extracellular space of the udder (Slettbakk *et al.*, 1995) and when severe may change the symmetry of the udder. It may change the orientation of the teats towards a lateral direction and increase the risk of damage on the teats by the bended position during the milking process (Slettbakk *et al.*, 1995; Waage *et al.*, 2001). Udder and teat oedema can impair the milk removal and be associated with retrograde flow (Fox, 2009). Additionally, an oedematous, rigid and enlarged in diameter teat is less likely to react normally to the milking machine, and may be more vulnerable to forces generated by changes in pressure during pulsation, due to reduced tissue elasticity. The decreased length of the teats caused by severe oedema of the udder can increase risk of liner slip, even when small liners are used (Slettbakk *et al.*, 1995; Waage *et al.*, 2001). Finally, udder oedema often contributes to impaired local blood circulation, which may decrease udder defence mechanisms (Waage *et al.*, 2001).

Excesive udder oedema is a significant risk factor for mastitis in heifers kept in pasture management systems (Slettbakk *et al.*, 1995; Waage *et al.*, 2001; Compton *et al.*, 2007b; McDougall *et al.*, 2009; Piepers *et al.*, 2009; Passchyn *et al.*, 2014). Heifers with moderate or severe udder oedema at parturition were 1.7 times more likely to suffer an episode of CM than heifers that with no or slight oedema (Waage *et al.*, 2001)Compton *et al.* (2007b). Teat oedema, as distinct from udder oedema, was also associated with increased risk of CM (odds ratio (OR) = 2.2) after correction for the effect of other significant factors, including udder oedema (Waage *et al.*, 2001). Udder oedema has also been associated with increased risk of IMI (Waage *et al.*, 2001; Passchyn *et al.*, 2014) and high SCC (Waage *et al.*, 2001; Santos *et al.*, 2004; Daniels *et al.*, 2007).

2.4.2.8 Age at calving

The age at first calving is associated with the risk of mastitis (Oltenacu and Ekesbo, 1994; Waage *et al.*, 1998; Waage *et al.*, 1999). Older heifers were 13% more likely to develop IMI or CM than younger animals, independent of the bacteria isolated (Wanner *et al.*, 1999; Bassel *et al.*, 2003). The underlying mechanism is speculative. Older heifers are heavier, which could affect ease of calving and increase postpartum sternal recumbency time due to dystocia (see Reproductive disorders, 2.4.2.10), and may have had a longer period of exposure to mastitis pathogens (Wanner *et al.*, 1999; De Vliegher *et al.*, 2012). Alternatively, age may be a proxy for other risk factors, such as season of calving in countries with pasture-based seasonal dairy industry, as for example in New Zealand (McDougall *et al.*, 2009).

2.4.2.9 Blood in the milk and milk leakage

Blood in milk was reported as risk factor, although the reason is not clear (Waage *et al.*, 2001; Fox, 2009). The presence of blood in the milk, close to parturition, has commonly been attributed to capillary bleeding due to congestion, for example in heifers with udder oedema, or to the rupture of a blood vessel by direct trauma (Waage *et al.*, 2001; Radostits *et al.*, 2006). Blood in the milk may stimulate the growth of bacteria, especially if IMI was already present, therefore increasing the risk of CM (Waage *et al.*, 2001). In their study, heifers with blood in the milk at parturition were 3.4 times more likely to

experience CM during the first two weeks of lactation than those without blood in the milk.

Milk leakage at calving has increases the risk of mastitis, probably because an open teat orifice can act as porte d'entrée for bacteria (Waage *et al.*, 1998; Fox, 2009).

2.4.2.10 Reproductive disorders

Reproductive disorders, such as retained foetal membranes, endometritis, pyometra, dystocia and twin births, increase the risk of IMI, CM or high SCC in heifers in early lactation (Barnouin and Chassagne, 2001; Svensson *et al.*, 2006; Fox, 2009; McDougall *et al.*, 2009; Waller *et al.*, 2009; Passchyn *et al.*, 2014). Dystocia in particular is associated with a high risk of CM or CNS infection within the first month postpartum (McDougall *et al.*, 2009; Passchyn *et al.*, 2014).

Four etiological explanations for the increase of CM in heifers with dystocia have been suggested (Barnouin and Chassagne; 2001): infection of the udder subsequent to uterine lesions contaminated by intestinal bacteria, especially coliforms, during calving management and posteriorly bacteraemia (Faye *et al.*, 1986); higher risk of IMI associated with an increased contact time with dirty bedding due to dystocia; impaired immune defences due to accentuated negative energy and protein imbalances, caused by decreased appetite in heifers with dystocia (Kehrli *et al.*, 1989); and, increased plasma cortisol induced by stress during dystocia, which can lead to a subsequent decrease in phagocytosis (Persson *et al.*, 1992; Mallard *et al.*, 1998). Whatever the mechanism may be, choice of appropriate sires to reduce the risk of dystocia in heifers should be part of an udder health management plan.

2.4.2.11 Genetic potential

The genetic potential for high milk production demonstrated by Holstein-Friesian heifers and other breeds has a strong association with higher potential for subclinical mastitis in early lactation in comparison to dual purpose heifers (Myllys and Rautala, 1995; Bludau *et al.*, 2014). Similarly, the Holstein-Friesian breed was identified as risk factor for postpartum subclinical mastitis (Myllys and

Rautala, 1995) and for CM in pasture-grazed systems, with a IRR of 1.94 (Compton *et al.*, 2007b).

Morphological characteristics such as good udder conformation with high set udders are associated with good udder health. Heifers with lower set udders have greater prevalence of subclinical mastitis, possibly due to a smaller distance to the ground, which may increase the risk of contamination of the teats with slurry splashes (Bludau *et al.*, 2014), water or mud. Low minimum teat height above the ground is associated with an IRR of 1.32 and 2.05 for postpartum subclinical and CM, respectively, in grazing heifers (Compton *et al.*, 2007b). An association between teat end to floor distance and subclinical and CM had also been demonstrated in non-grazing systems (Slettbakk *et al.*, 1990; Slettbakk *et al.*, 1995).

3 Use of infrared thermography in veterinary diagnostics

Infrared thermography has been used for a long time for military and building proposes. In the last decades, it has also been used to diagnose diseases or conditions through non-invasive or contactless detection of temperature changes caused by inflammatory processes (Usamentiaga *et al.*, 2014). This approach is very appealing for the dairy industry, especially for udder health screening of heifers, because it might allow for mastitis detection without the need to collect samples and the associated risk of causing IMI and mastitis.

3.1 Infrared thermography principles

The use of IRT as diagnostic tool involves the production of an image, the thermogram (also known as thermal image or IRT image), which can be displayed in grey or colour-scale and viewed in the camera display or using computer software. In those images, each pixel corresponds to an individual temperature. This temperature (heat) is detected by the thermal camera as infrared radiation, characterized by a wavelength of 8 to 12 μm that is emitted by the source or surface and is calculated using the Stefan-Boltzman Law ($R = \varepsilon\sigma T^4$, where R is the infrared radiation measured in W/m^2; T is the computed temperature measured in K; ε is the emissivity of the surface and σ is the Stefan-Boltzman constant 5.67 x 10^{-8} $W/m^2/K^4$) (Speakman and Ward, 1998; Berry *et al.*, 2003; McCafferty, 2013; Church *et al.*, 2014). Thermal cameras can produce very sharp images, i.e. images with so-called microcontrast between individual pixels as opposed to overall image contrast, with a precision of 0.08°C (Stelletta *et al.*, 2012).

The application of this technique to veterinary medicine and other fields of biology is based on the fact that skin temperature reflects the underlying blood circulation and tissue metabolism (Jones and Plassmann, 2002; Ring and Ammer, 2012), and therefore changes in the thermal pattern might reflect inflammatory processes and other disease processes that can affect the microcirculation of the skin (Stelletta *et al.*, 2012). The use of the technique for veterinary studies relies on the high emissivity of the skin, which ranges from 0.93 to 0.98,

depending on the coat length and density (Stelletta *et al.*, 2012). Emissivity has been defined as the ability of a surface to emit or absorb radiation, which for a biological tissue is close to a blackbody: the perfect emitter (ε=1) that absorbs all radiated heat (McCafferty, 2013).

IRT cameras are most accurate when used within a few meters from the animals, and when the mean ('min' and 'max') temperature can be recorded from a defined area highlighted by a laser guide (McCafferty *et al.*, 2005). Several factors influence image quality and need to be identified and if possible controlled to improve image interpretation (Rekant *et al.*, 2016). For example, it is important to collect the thermograms out of direct sunlight, rain and wind drafts, and an animal's hair coat must be free of dirt, moisture or foreign material (Stewart *et al.*, 2005; Poikalainen *et al.*, 2012; Okada *et al.*, 2013).

3.2 Overview of infrared thermography applications in ruminants

The increased interest in IRT and its application in research and medical fields of production animals, are not just due to the diagnostic potential of this tool (Stelletta *et al.*, 2012), but also to its possible usefulness for preventive medicine (Bortolami *et al.*, 2015). IRT has been described as a desirable tool to obtain surface temperatures because of its non-invasive and non-contact character (McCafferty, 2013), avoiding undue stress to the animal and the use of sedation. However, its cost was one of the main hindrances to wide use of this technology. Thermal imaging cameras are now more affordable (<£1000 - >£30,000) and there is a higher diversity to suit different needs (McCafferty, 2013; McCafferty *et al.*, 2015). It is considered extremely useful in cases of large herds or flocks, where obtaining body temperature of each animal with rectal thermometer can be difficult and time consuming (Rekant *et al.*, 2016).

The first studies using IRT in cattle were conducted in the 1980s when Hurnik *et al.* (1984) described IRT as a suitable tool for detection of health disorders in Holstein-Friesian dairy cattle, with the temperature of the gluteal region increasing above 37°C within three days prior to the detection of clinical

disease. Their study was designed for a different purpose, i.e. to detect changes in skin temperature related with oestrus, and the specific use of IRT for disease detection was not assessed. No effort was made to correct for the influence of oestrus or barn environmental temperature on the gluteal area. The number of diseased animals was small (n= 16) and included animals affected by several conditions (pneumonia, mastitis, kidney infection and minor swellings and injuries). The methodology required the collection of polaroid photographs from the video system connected to a portable IRT camera and had lower thermal sensitivity than modern IRT cameras.

Nowadays, IRT is used for disease detection rather than the detection of oestrus (Poikalainen *et al.*, 2012), due to the potential for early identification of increases in body temperature of individual animals that can indicate the development of fever or local inflammation without the need for capture or restraint of the animals (Rekant *et al.*, 2016). The potential of IRT as diagnostic tool has been explored for assessment of welfare, reproduction, production, lameness, infectious diseases and udder health, as detailed in the subsequent sections.

3.2.1 Infrared thermography applied to non-diseased animals

3.2.1.1 Normal body thermal distribution pattern

The surface temperature varies between different parts of the bovine body. Poikalainen *et al.* (2012) reported that healthy and normal body skin of dairy cattle has a temperature between 27.4°C and 28.2°C. Based on the thermal profile of the left side of the body, the highest temperature is found in the udder (average surface temperature = 30.7°C) followed by the eye (average surface temperature = 28.6°C) (Poikalainen *et al.*, 2012). Eye temperature was not affected by environment temperature (Gloster *et al.*, 2011) and of all anatomical regions with surface temperatures measured by IRT, the eye is considered the most consistent and reliable indicator of the body temperature in sheep and cattle (Gloster *et al.*, 2011; Poikalainen *et al.*, 2012; George *et al.*, 2014; Salles *et al.*, 2016). Udder surface temperature is affected by parity, pregnancy and stage of lactation (Sathiyabarathi *et al.*, 2016).

The distal part of the legs had the lowest temperatures and were the anatomical region most affected by the environmental temperature, with the coronary band showing the highest temperature and the hoofs lowest temperature (Poikalainen *et al.*, 2012). Temperature of healthy feet ranges from 17.2°C to 28.7°C (Main *et al.*, 2012; Poikalainen *et al.*, 2012). Healthy hoof temperature is influenced by cattle activity, with individual variations during the day (Gloster *et al.*, 2011).

3.2.1.2 Welfare

Skin temperature changes are a proportional indicator of stress that can be measured non-invasively by IRT. They are congruent with hormonal and behavioural reaction to acute stress in hens (Herborn *et al.*, 2015) and have been used to measure tissue damage caused by hot-iron and freeze brand identification, and the associated discomfort in cattle. IRT was used to assess the extent and duration of inflammation and indicated that the damage persists until the formation of a new collagen matrix and reestablishment of the blood supply at the site of branding (Schwartzkopf-Genswein and Stookey, 1997). Later, Stewart *et al.* used IRT to assess welfare through changes in heat production and heat loss caused by physiological processes activated during situations of stress. Eye temperature measured by IRT could be used to detect pain and fear associated with routine practices, such as disbudding without anaesthesia and cattle handling with electric prods, hitting and shouting, with a drop in eye temperature in response to stress (Stewart *et al.*, 2008; Stewart *et al.*, 2008). This drop in eye temperature in the face of stressful situations was also observed in sheep, although the mechanism responsible for this change in temperature was not clear (Stubsjøen *et al.*, 2009). Measurement of eye temperature can contribute to improvement in welfare, animal health and production through early intervention by the farmer (Stewart *et al.*, 2017). However, it is important to remember that there are substantial differences in the response to stress among species, which can result in different eye temperatures (Rekant *et al.*, 2016).

Recently, IRT has been validated as a reliable method to measure respiratory rate in dairy cattle and it was suggested to have potential for early detection of metabolic diseases (e.g. bloat, rumen acidosis), distress and discomfort, when

combined with other IRT monitoring (Stewart *et al.*, 2017). The ability to accurately estimate the respiratory rate by IRT was also found in studies with humans (Lewis *et al.*, 2011).

3.2.1.3 Production

Feed intake and feed conversion efficiency are highly important in livestock production because of the positive correlation between the weight gain per unit of feed consumed and economic returns (Rekant *et al.*, 2016). Body surface temperature measured by IRT accounted for more than 70% of residual food intake variation (Montanholi *et al.*, 2010) and it was correlated with feed conversion efficiency traits in dairy and beef cattle (Montanholi *et al.*, 2008, 2010). For example, more efficient steers presented cooler body extremities when compared with less efficient steers (Montanholi *et al.*, 2010). Thus, IRT was suggested as an important tool to identify cattle with low residual food intake, which have desirable genetics because of their higher capacity of convert food into meat (Montanholi *et al.*, 2009; Rekant *et al.*, 2016). Temperatures of the left flank of cattle measured by IRT were positively correlated with the production of methane after feeding, revealing the potential of IRT to estimate methane emissions, which is of growing environmental concern in the cattle production industry (Montanholi *et al.*, 2008).

IRT could also detect changes in vulvar skin temperature indicative of oestrus (Talukder *et al.*, 2014; Radigonda *et al.*, 2017), although it has poor performance in detection of cows' ovulation time (Talukder *et al.*, 2015). The sensitivity of IRT to indicate oestrus is greater than the sensitivity of visual observation (67%) and aids for heat detection (estrotect activation; 67%), i.e. a threshold of 1°C reaches 92% *Se* whereas a threshold of 1.5°C reaches 75% *Se*. However, the use of IRT overpredicts the incidence of oestrus due to its lower specificity and positive predicted values (*Sp* of 29% and 57%, and positive predicted values of 64% and 69% for 1°C and 1.5°C thresholds, respectively) (Talukder et al., 2014). Additionally, vulvar IRT temperature presents the greatest specificity (80%) to detect ovulation time when compared with IRT temperatures of other individual body areas to detect ovulation time, however its sensitivity is the poorest (21% vs for example 56.8% *Se* of muzzle temperature) (Talukder *et al.*, 2015). It can be also used to assess scrotal

temperature gradients of bulls used for reproduction and evaluate the effect of environmental stress on bulls reproductive parameters (Menegassi *et al.*, 2015), safely for the observer.

Later in the production chain, IRT can be used to check meat quality. The methodology described by Tong *et al.* (1997) involves IRT scanning of cattle before slaughter to detect animals that are likely to yield dark, firm and dry meat after slaughter. The method was suggested to be particularly useful to detect high probability of poor meat quality in groups of unstressed animals. The rejection of animals is based on the measurement of their central tendency temperatures. If these differ from the central tendency temperature of the group by more than 0.9 standard deviation (or 1.28 times the standard deviation if mean temperature is used) those animals are rejected (Tong *et al.*, 1997).

3.2.2 Infrared thermography applied to diseased animals

3.2.2.1 Lameness

IRT has been used fairly routinely for detection of lameness in horses (Turner, 1991; Eddy *et al.*, 2001). The use of this technology to identify lame cattle is also a reality in bovine medicine (Nikkhah *et al.*, 2005; Alsaaod and Büscher, 2012; Stokes *et al.*, 2012). IRT was successfully used to identify the focus of inflammation in a heifer with septic arthritis and to provide additional information about a neurovascular injury in a bucking bull, showing the utility of this technology as aid in the diagnosis and development of a treatment plan (Cockcroft *et al.*, 2000; Caldwell *et al.*, 2017). Measurement of coronary band temperature is useful for early diagnosis of laminitis or claw lesions (e.g. sole haemorrhages), particularly during the early and middle stages of lactation (Nikkhah *et al.*, 2005; Alsaaod and Büscher, 2012). Coronary band temperature is not significantly increased in cases of digital dermatitis, whilst skin temperature of the feet is (Alsaaod *et al.*, 2014). The sensitivity and specificity of the feet skin temperature to detect digital dermatitis with a cut-off of 0.99°C was 89.1% and 66.6%, respectively (Alsaaod *et al.*, 2014). This increase in foot temperature in feet with lesions was also observed by Wood *et al.* (2015). Sole temperature has also been evaluated by IRT as way of checking for corium inflammation (Oikonomou *et al.*, 2014). Sole temperature increased as digital cushion

thickness decreased, and correlated well with locomotion scores prior to the detection of visible claw lesions in early lactation (Oikonomou *et al.*, 2014).

Detecting feet with increased temperature is important to help prioritise individual cattle with feet problems within the herd for early treatment (Main *et al.*, 2012; Stokes *et al.*, 2012). At the same time, it minimises the amount of handling required to identify affected animals, thus reducing stress and workload for cattle and people (Main *et al.*, 2012; Stokes *et al.*, 2012).

3.2.2.2 Infectious diseases

Early identification by IRT of animals with abnormal increased body temperature allows for monitoring of individual animals, followed by isolation, testing and treatment of pyrexic animals during outbreaks of diseases. This helps in the control of the transmission of disease to the susceptible animals, increases the probability of successful treatment and reduces the economic impact of disease (Rekant *et al.*, 2016). A study with calves inoculated intranasally with BVD virus showed that temperatures of the orbital area captured by IRT were a highly sensitive indicator of infection several days prior to the onset of the clinical signs (Schaefer *et al.*, 2004). A similar response was found in calves with respiratory disease, and IRT can be used to identify calves in early stages of respiratory disease (Schaefer *et al.*, 2007, 2012). On the day of visual detection of naturally affected BRD, the positive and negative predictive value of maximum orbital temperature elevation as measured by IRT was 86% and 67%, respectively (Schaefer *et al.*, 2007). The low negative predicted value reflects the limited sensitivity of the method. Recently, the same research team reported an improvement of IRT predicted values on the day of visual BRD detection, i.e. positive predicted value of 87% and negative predicted value of 100% (Schaefer *et al.*, 2012). The main difference between studies relies on the methodology of IRT data collection, which was performed by an automated remote sensing system (infrared scanning station) that allowed an increased frequency of data collection per animal. As a predictor for BRD four to six days prior to its visual detection, *Se* and *Sp* were only 54% and 68%, respectively (Schaefer *et al.*, 2007). Thus, despite significant associations between temperature and health status, use of IRT has limited value as a diagnostic test for this condition. This application of IRT resembles the use of this technology

for fever screening of passengers at airports and hospitals during the outbreak of severe acute respiratory syndrome in East Asia (Chiu *et al.*, 2005; Chiang *et al.*, 2008; Ring and Ammer, 2012; Sun *et al.*, 2017). The recent vital-sign-based IRT screening test, which includes facial skin temperatures and respiratory rates measured by monitoring the temperature around the nose by IRT images, has higher sensitivity than commonly used IRT screen methods for febrile passengers (sensitivity of 87.5% and negative predictive value of 91.7% versus 23 to 29% sensitivity for standard methods such as ear drum and digital infrared thermal imaging) (Sun *et al.*, 2017). Similar improvements in IRT methodology may also enhance its value as diagnostic test in veterinary medicine.

IRT was validated to measure sheep body temperature after experimental infection with bluetongue virus. Eye temperature was strongly correlated with rectal temperature, allowing detection of pyrexic sheep with a sensitivity of 85% and specificity of 97% (Pérez de Diego *et al.*, 2013). Food-and-mouth disease (FMD) is another infectious disease that has been studied with IRT during experimental infection. The coronary band temperature can increase 24 to 48 hours before vesicular lesions become visible (Rainwater-Lovett *et al.*, 2009). Even though not a specific sign of FMD (see Lameness, 3.2.2.1) IRT may be of use during the control, eradication and recovery phases of an FMD outbreak, when combined with other rapid diagnosis tests (Rainwater-Lovett *et al.*, 2009). IRT has also been evaluated as diagnostic tool for systemic bacterial infections. Its use allowed veterinarians to shorten the time needed for screening of animals for tuberculosis. It reduced the time between injection of *Mycobacterium* antigen and the reading of the local inflammatory response from 72 hours to 24 hours when using a threshold of 37.5°C. In addition to shortening the time to diagnosis, IRT improved sensitivity from 80% to 86% compared to skin thickness measurements (Johnson and Dunbar, 2008).

3.2.2.3 Udder health

A healthy bovine udder presents an uniform thermal image, reflecting the uniformity of the blood supply within the skin surface (Gebremedhin *et al.*, 2013). Bitman *et al.* (1984) showed very high correlation (r = 0.98) between udder temperature and internal body temperature in lactating dairy cows, with temperatures of 38.8°C ± 0.1°C. Several studies have demonstrated a circadian

fluctuation of the body temperature in dairy cattle (Wrenn *et al.*, 1961; Bitman *et al.*, 1984; Araki *et al.*, 1987; Lefcourt *et al.*, 1999). Whilst udder temperature follows this circadian fluctuation (Berry *et al.*, 2003), the daily variation in udder temperature is smaller than the rise in temperature resulting from an induced mastitis response, suggesting that differentiation between normal and abnormal temperature increases should be possible (Kunc *et al.*, 2007). The early onset of mastitis is characterised by an inflammatory response and commonly causes an associated increase of temperature in the quarter affected (Berry *et al.*, 2003). Therefore, IRT can potentially have merit as an early, rapid and real-time method of detecting mastitis caused by IMI in dairy cows (Bortolami *et al.*, 2015), provided that variation in the environmental temperature, ambient humidity, physiological state and productivity of the animal, exercise level, and time relative to milking and feeding are taken into account (Knizkova *et al.*, 2007; Kunc *et al.*, 2007; Colak *et al.*, 2008; Rekant *et al.*, 2016). In animals around calving should be considered that the increase in capillary permeability might cause plasma leaks into the interstitial tissue and lead to pronounced oedema (McGavin and Zachary, 2007). This may be of particular relevance in heifers, where incidence and severity of oedema is greater than in cows (Kojouri *et al.*, 2015).

IRT has been used in humane medicine for diagnosis of mammary pathologies (Frize *et al.*, 2003; Lahiri *et al.*, 2012), and it has been evaluated as tool for detection of clinical and subclinical mastitis in lactating cows. In 1984, Hurnik et al. were able to detect 4 out of 6 mastitis cases in dairy cows with the application of IRT. More recently, Scott *et al.* (2000) experimentally induced CM with the infusion of *Escherichia coli* endotoxin into the mammary gland, and measured an increase of 2.3°C in the udder temperature with IRT images of the caudal aspect of the udder, with IRT detecting heat and swelling associated with mastitis earlier than traditional measures. Pezeshki *et al.* (2011) detected a similar rise in temperature (2 to 3°C) but that increase was delayed in relation to the rise observed in rectal temperature. Hovinen *et al.* (2008) used the same endotoxin to experimentally induce CM. They reported a smaller but significant increase of the temperature of the quarter surface (1 to 1.5°C) in infused compared to non-infused quarters. In contrast to Scott *et al.* (2000), they observed local signs in the infused quarters prior to the increase of the udder

skin and rectal temperature. The delay in detection of an increased udder skin temperature was explained as a possible result of the vasoconstriction of peripheral blood vessels that occurs during the systemic effects of the *E. coli* endotoxin, and of the pronounced oedema of the quarters which caused impaired blood circulation and decrease of the temperature of the udder skin (Hovinen *et al.*, 2008). Metzner *et al.* (2015) have also found a significant decrease in the average temperature in infected quarters, which was also considered to be the result of the development of oedema. Thus, mastitis in cattle may be associated with increased or decreased skin temperature. In dairy ewes that are challenged with *E. coli* endotoxin, IRT was unable to differentiate between subclinically or clinically infected and healthy udder halves (Castro-Costa *et al.*, 2014).

IRT was also suggested as a tool to screen dairy cows for subclinical mastitis, allowing a non-invasive early detection and potentially contributing to the reduction of production losses (Colak *et al.*, 2008; Polat *et al.*, 2010). Udder skin surface temperature and CMT results of each quarter were strongly correlated (Colak *et al.*, 2008; Polat *et al.*, 2010), with an increased average surface temperature in response to an increase in degree of CMT reaction, which is a semi-quantitative measure of SCC (Colak *et al.*, 2008). This positive correlation was also found between skin surface temperature and actual SCC measurement, with an increase in skin surface temperature of 2.35°C in quarters with inflammation (SCC >400,000 cells/ml) compared to healthy quarters (SCC ≤400,000 cells/ml) (Polat *et al.*, 2010). The sensitivity and specificity of IRT and CMT to detect quarters with high SCC were similar (95.6% and 93.6% for IRT, and 88.9% and 98.9% for CMT, respectively), with no difference in the area under the ROC curve (Polat *et al.*, 2010). The correlations between udder skin surface temperature and CMT and SCC were also found in dairy camels (Samara *et al.*, 2014). In sheep, IRT also showed higher temperatures glands with subclinical mastitis (Martins *et al.*, 2013). Thus, IRT was considered to have high predictive diagnostic value for subclinical mastitis, when microbiological culture is not available, with the advantage of being non-invasive (Colak *et al.*, 2008; Polat *et al.*, 2010; Martins *et al.*, 2013; Samara *et al.*, 2014). However, using a different definition of subclinical mastitis based on SCC (>200,000 cells/ml) and culture (positive, non-contaminated sample), Porcionato *et al.* (2009) found no

association between SCC and udder skin temperature nor differences in udder skin temperature in relation to the type of bacteria isolated from the milk samples (Porcionato *et al.*, 2009). The lack of association between results in this study is probably related with differences in research methodology, such as milk sampling prior to IRT image collection (which would affect IRT temperature readings), the use of a lower SCC threshold (which can represent an early phase or less pronounced inflammatory response, which is less likely to be associated with the higher blood perfusion and hence higher udder skin temperature commonly seen in cases of more active inflammation accompanied by high SCC), and the use of bacteriological criteria rather.

In addition to udder health, the milking process has been studied by IRT. Overmilking and liner type can affect teat temperature, and tend to increase the teat wall thickness and teat canal length The variability in teat length is highly affected by the pre-milking preparation, which explains approximately 80% of the variation, whilst teat temperature seems to be more dependent of the type of liner (Paulrud *et al.*, 2005). With proper pre-milking technique, the surface temperature of a healthy udder is minimally affected by the milking process (Poikalainen *et al.*, 2012). Thus, IRT monitoring of the udder before and after milking could potentially help to monitor the negative impact of suboptimal milking technique or liners on the teats. Early detection and intervention could prevent an increase in the mastitis incidence due to poor liner or machine adjustment or use.

Despite its limitations, IRT has been useful for detection of CM, high SCC or IMI in several studies. In pre-calving heifers, IRT appears to be the only non-invasive method to do this. Therefore, it deserves further investigation. In addition, as dairy herds expand, they are more likely to have specialized heifer rearing units where IRT based udder health screening could be incorporated in the management routine, making it even more attractive as a diagnostic tool. Including IRT within the group of technology of renewing interest in point-of-care diagnostics and precision farming, which aim to support sustainable intensification of the dairy industry and reduce the use of antimicrobials and the risk of antimicrobial resistance.

4 Aim of the study

This cross-sectional study was conducted with the aim to evaluate the usefulness of IRT under field conditions as a non-invasive early detection tool for abnormal udder health caused by intramammary infection during the last trimester of pregnancy in nulliparous dairy heifers.

Research hypothesis:

The study has been performed under the hypothesis that IMI would cause an inflammatory response (mastitis) with increased blood flow circulation to the affected quarter(s), leading to an increase in udder skin surface temperature which could be detected at distance by IRT.

Objectives:

1. To evaluate the practicality of IRT to screen heifers' udder skin temperature on-farm;
2. To evaluate the health status of nulliparous heifer mammary glands at calving;
3. To evaluate species level causes of intramammary infection on the first day of lactation;
4. To investigate the use of IRT as predictor for the udder health at calving.

5 Material and methods

5.1 Regulatory compliance

This research was conducted with approval from the Ethics & Welfare Committee of the University of Glasgow – School of Veterinary Medicine, which included an ethical review of the use of animal subjects, material and data (Ref31a/14). Written consent from the farmer was also obtained prior to the beginning of the study.

5.2 Farm overview

The study was conducted on a single commercial dairy farm of 700 lactating Holstein-Friesian cows in central Scotland, with a previous history of heifers calving with subclinical and clinical mastitis. The herd was milked in a 52-point rotary parlour three times a day. Freshly calved animals are the first group being milked, followed by the first lactation, high yield and mid yield groups. The herd had a mastitis control program in place, which was monitored closely through monthly data review, SCC measurements, microbiological analysis of milk samples from CM and subclinical mastitis and staff meetings. However, nulliparous heifers were not included in the mastitis control program.

5.2.1 Nulliparous housing and management

Between nine and twenty-two months of age, nulliparous heifers were housed in a contracted farm distant from the main unit.

The reproductive protocol started at thirteen months of age, with the animals being artificially inseminated with sexed semen at heat detection. In case of reproductive acyclicity they were synchronized with Co-synch protocol and fixed time inseminated with Fertility Plus Easy Calver semen. The same protocol was used in cycling heifers that failed to conceive after three inseminations with sexed semen. All animals were submitted to two ultrasonographic examinations for pregnancy diagnosis (PD): first PD around 28 days post-service and second PD

around 60 days post-service. No animals were bred after eighteen months of age.

During the winter, heifers were maintained indoors, in rubber mattress cubicles, which were bedded with sawdust and cleaned with manual scrapers. Manure removal from the passages was done by automatic scrapers. Heifers were fed with grass silage and a blend of rolled cereals, with *ad libitum* access to water. In late spring, if the weather allowed, some groups had access to pasture: the group formed by heifers between nine months old (age of arrival at the heifer unit) and twelve months old (pre-bulling/breeding age), and the group composed of pregnant heifers that had a positive second pregnancy diagnosis.

Two months before the estimated calving date the animals were moved to the main unit and housed in a heifer group. Two to three weeks before calving they were moved to a close-up and calving pen dedicated only to heifers that was bedded every morning and mucked out every three weeks. They were fed with a transition total mixed ration and had *ad libitum* access to water. After calving and shortly after being moved to the fresh pen, they were introduced into the group of first lactating animals in the milking shed. The farmer has reported an average of between 10% and 15% of heifers starting lactation with mastitis (including clinical and subclinical mastitis) in the previous years.

5.3 Animals and experimental procedure

5.3.1 Protocol overview

Data collection started in October 2014 and was completed in March 2016, with the last heifers enrolled in the study finishing their first lactation. The diagram illustrates the main data collection points of the study (Fig 5-1). Collection of the first set of IRT images took place from November 2014 to March 2015 and the second set was collected from December 2014 to May 2015. The first set of images, collected approximately 2 months prior to estimated calving date, reflected the pre-colostrogenesis period whereas the second set of images was collected at approximately 2 weeks prior to estimated calving date, i.e. during the colostrogenesis period.

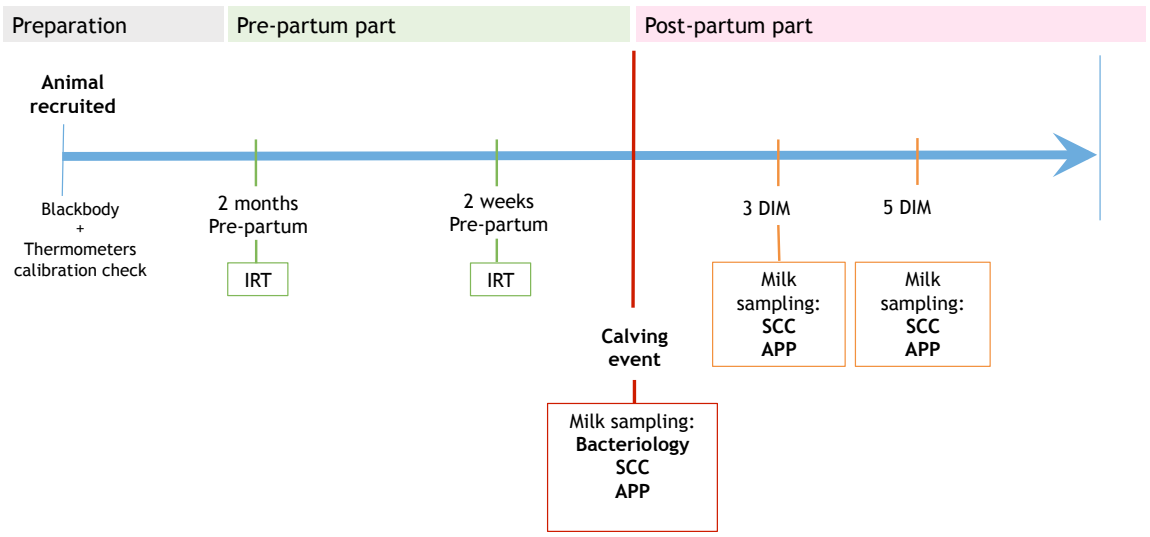

Figure 5-1 Diagram summarising the study protocol. APP - acute phase protein; DIM - days in milk; IRT - infrared thermography; SCC - somatic cell count.

All animal health events affecting animals on the study were recorded in herd management software DairyComp 305 (Valley Agricultural Software, Tulare, CA) and exported to an Excel file (Microsoft® Excel 2010, Microsoft Corporation, Redmond, WA).

5.3.2 Animal recruitment and equipment testing

5.3.2.1 Animal recruitment

Heifer recruitment was facilitated by the use of the herd management software DairyComp 305. In October 2014, 128 heifers confirmed in calf and with estimated calving date between January 2015 and May 2015 were listed for potential recruitment. From those, 126 clinically healthy non-lactating Holstein-Friesian heifers were recruited in the seventh month of pregnancy. Two heifers were excluded before the beginning of the study, with one animal confirmed not pregnant and the other euthanized after injury.

5.3.2.2 Blackbody

To ensure accurate calibration of the infrared camera on-farm, a cylindrical matte blackbody was built with a thermometer probe introduced in the centre, using a plastic bucket painted with matt black spray paint (Matt Super, Valspar Corporation, PlastiKote, UK) (Fig 5-2A). The thermometer probe (Digital

fridge/freezer alarm thermometer, Electronic Temperature Instruments Ltd, UK) measured over a range of -49.9°C to 69.9°C, with a resolution of 0.1°C and accuracy of ±1°C. Its calibration was checked against a mercury thermometer by submersion into water, which was submitted to an increase of temperature within the range of environmental temperature expected in the shed from November to May (3°C to 20°C) (Fig 5-3).

Figure 5-2 Instrument preparation for IRT image collection. A - Blackbody; B - Humidity and temperature gauge.

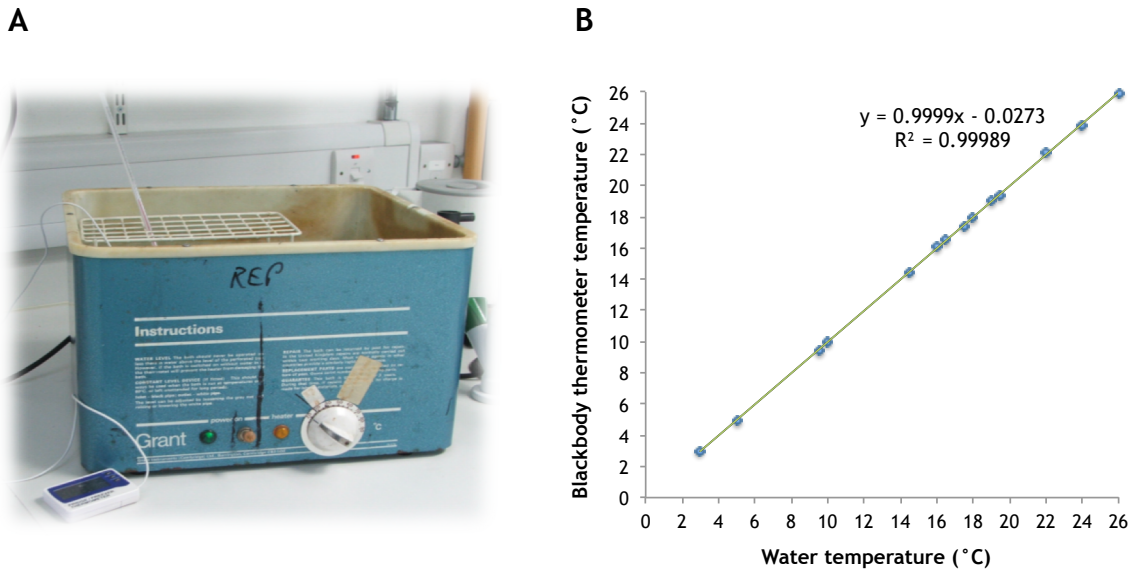

Figure 5-3 Blackbody thermometer calibration check: water bath in the laboratory (A) and scatterplot summarising calibration check results (B).

5.3.2.3 On-farm tests using the infrared camera

The hand-held infrared camera (ThermaCAM™ E300 IR, FLIR systems Ltd, UK) with a spectral range of 7.5 to 13 μm, accuracy ±2 % and thermal sensitivity <0.1 °C, was trialled twice at the Glasgow University's Cochno farm to check the practicality and feasibility of the procedure to capture images at different projections of the udder and to define the protocol for IRT collection at the commercial farm. This trial involved six nulliparous heifers at different stages of gestation, which were restrained in similar conditions to those at the commercial farm, i.e. using head yokes or a cattle crush. The camera had been calibrated by the manufacturer and was serviced once a year.

5.3.3 Infrared thermography

5.3.3.1 Animal handling and instrument preparation

All the animals in the study were assessed in standing position after twenty to thirty minutes of resting, either in a cattle crush after resting in the cattle race or in the head yokes. Assessment was conducted prior to routine procedures, such as footbathing, deworming, vaccination, tail clipping or at the same time as bedding management procedures and animal movements between groups (for example going into the close-up group), which required maintaining the heifers in the holding yard. This minimized the need for animal handling specific for this study reducing welfare impacts and distress.

A humidity and temperature gauge (Benetech LCD, Benetech, California, USA) was placed in the shed near the heifers (Fig 5-3). The gauge sampled at a rate of 2.5 times per second and measured temperatures within a range of -10°C to 50°C, with accuracy of ± 1°C and resolution of 0.1°C. Its relative humidity measurement range was from 5% to 98%, with accuracy of ± 3% (in the 30 to 95% relative humidity range) and resolution of 0.1%. Ambient temperature and relative humidity of the shed were registered immediately before and monitored until the end of the thermal image collection. This data was used to set the definitions of the thermal camera at the start of each thermography session and introduced in the software for IRT image analysis.

The blackbody was exposed to the shed environment temperature during the same length of time that the heifers were resting prior to image collection and

used to check the infrared camera calibration through the comparison of the temperature displayed at the thermometer of the blackbody and the temperature measured by the infrared camera of the centre of the blackbody, using an emissivity of one. Additionally, a digital laser measure (PLR 15 digital laser measure, Bosch, Germany) with measurement range of 0.15 to 15 m was fixed to the infrared camera at the same level as the lenses to measure the distance between the camera and the udder of the heifers.

With the heifers in standing position, their tail was gently held, and digital photographs from the freeze brand and udder of each animal were taken, in the same sequence and projections as described below for IRT images, using a digital camera (Finepix S5500, Fujifilm, Japan), as a visual aid to animal identification and additional guidance of the udder limits during analysis of IRT images.

5.3.3.2 Thermographic image collection

The initial protocol included three projections of the udder for each set of IRT images: caudocranial, ventrodorsal and lateral. However, due to the small size of the front quarters and management/restraint methods on the farm (cattle crush laterally fixed to the wall and heifers side-by-side in the head yokes), it was reduced to two projections, caudocranial and ventrodorsal.

The caudocranial projection was taken with the camera perpendicular to the back quarters and dropping lines from the hip pins passing via the hocks to the floor (Fig 5-4). Ventrodorsal projections were taken with an angle of 45°degrees with the drop lines hip pin-hock-floor (Fig 5-5). Both projections were taken at 70 cm from the udder. The digital laser meter was used to measure the distances. All IRT images were taken between 11 am and 2 pm, to control for the effects of the circadian rhythm. The emissivity of the thermal camera was set at 0.96, and all image collection was conducted inside the building, without wind drafts, reflective surfaces or direct sunlight interference.

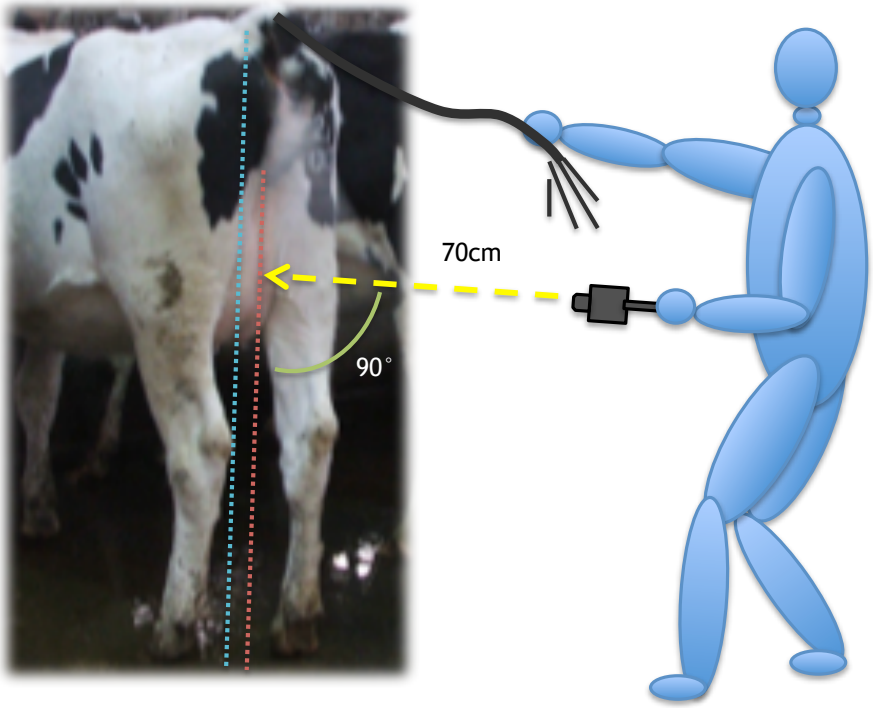

Figure 5-4 Caudocranial projection schematic presentation.

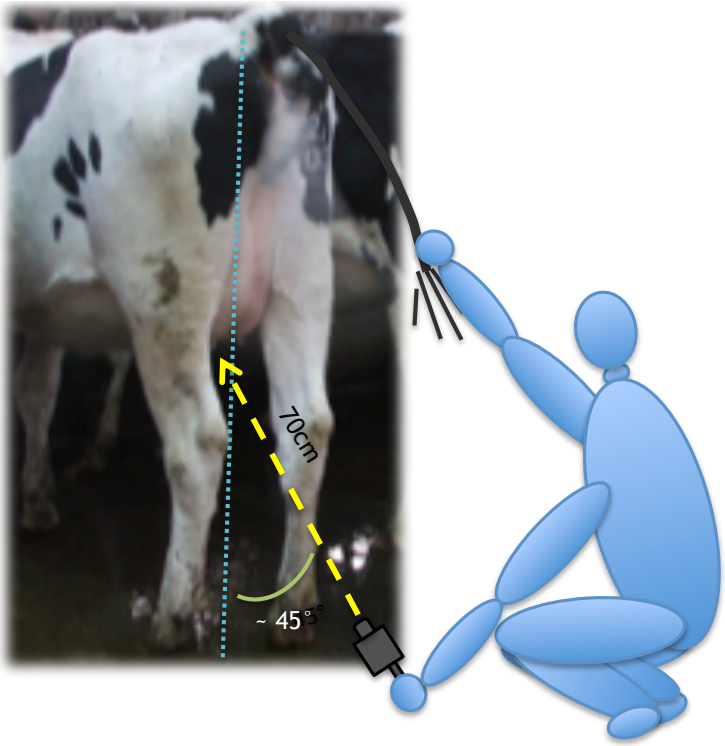

Figure 5-5 Ventrodorsal projection schematic presentation.

After collection of each set of IRT images, the rectal temperature of each animal was taken with a digital thermometer (Veterinary digital equine thermometer, Henry Schein, UK), followed by udder palpation to assess possible signs of mammary gland inflammation (differences of skin temperature, presence and consistency of nodules in any of the quarters). All information, including the presence of oedema, warts or supernumerary teats, was recorded (Appendix 2).

5.3.3.3 IRT image analysis

The single best image from each projection of each set of images, as described by a better udder position and contour definition in grey palette, was analysed using ThermaCAM™ Researcher Pro 2.10 (FLIR System, Danderyd, Sweden) software. Each image was adjusted within the analysis software for ambient temperature, humidity and distance to the udder.

Different geometric software-tools were used for image analysis. With the IRT images displayed on the grey palette, polygons were applied to all udder surfaces, including teats in the ventrodorsal projection, individual quarters and teats. Lines were applied to individual quarters and teats and 'spot meter' was used to assess the temperature of the teat meatus. Polygons were measured using manual tracing with a digital tablet for drawing (Intuos Pro creative pen tablet, Wacom, Japan). Areas were drawn based on their contours, with the contour of the area being done internally at approximately ±3 mm from the anatomical area contour, to avoid the area of higher curvature (Fig 5-6, Fig 5-7). For polygon identification of individual quarters, intermammary grooves were also taken as reference. After the polygons tool had been applied, the Lines tool was used to draw dorsoventral lines through the centre of the polygons. This was done for caudocranial projection as illustrated in Fig 5-6 and for ventrodorsal projection as illustrated in Fig 5-7. Descriptive parameters for each polygon, such as minimum ('min'), maximum ('max'), average ('avg'), difference between maximum and minimum ('max-min') and standard deviation were obtained automatically through ThermaCAM™ Researcher Pro 2.10 software for each area, line and spot defined by the geometric tools. The author conducted all image analyses (n = 470; n = 238 at 1st IRT – 119 IRT images of caudocranial projection and 119 images of ventrodorsal projection; n = 232 at 2nd IRT – 116

IRT images of caudocranial projection and 116 images of ventrodorsal projection).

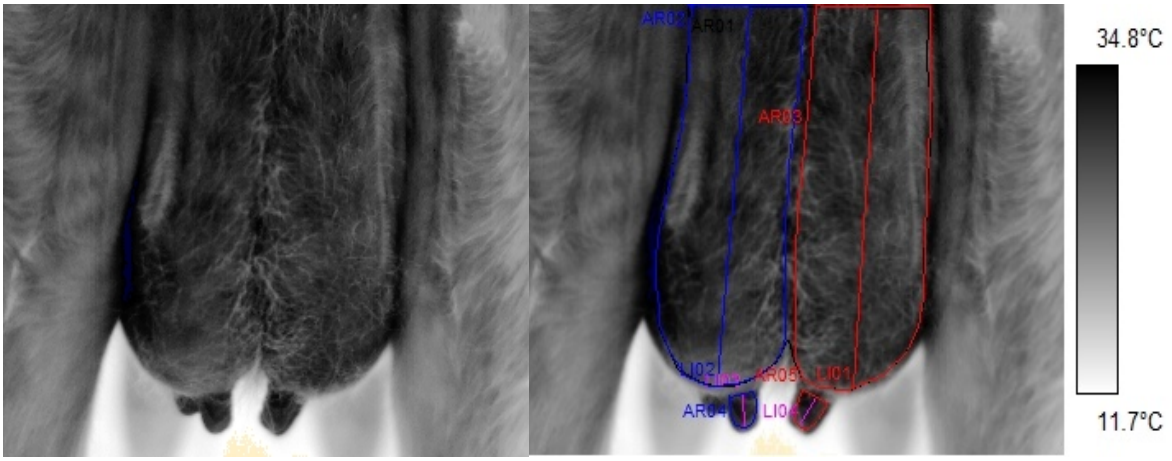

Figure 5-6 IRT image analysis: caudocranial projection. AR – area defined by polygons tool; LI – line defined by lines tool.

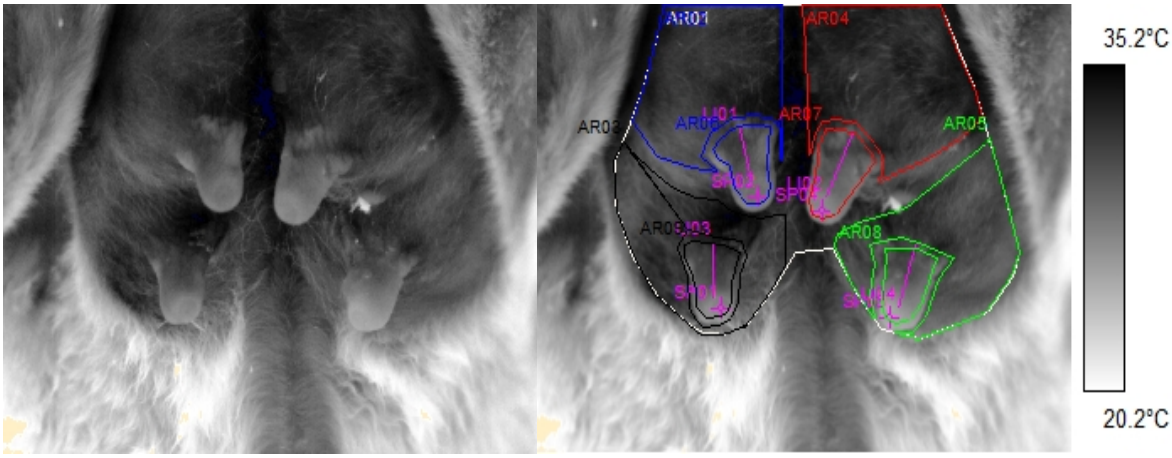

Figure 5-7 IRT image analysis: ventrodorsal projection. AR – area defined by polygons tool; LI – line defined by lines tool; SPO – spot defined by 'spot meter' tool.

5.3.4 Milk sampling

Milk sample collection took place over a period of six months, from January 2015 to June 2015. Quarter milk samples were collected according to the National Mastitis Council (NMC) recommendations, with minor modifications (Fig 5-8). Colostrum or milk samples were collected by the farmer following the sampling collection technique protocol and after a training session with the author.

Colostrum samples were collected aseptically from each quarter of one hundred and eleven animals at the first day of lactation. The first sample from each quarter was collected into a pre-labelled 15 ml sterile centrifuge tube (VWR international Ltd, UK) (Fig 5-9A) and stored immediately at -20°C at the farm. Once a week, samples were transported at the same temperature (-23°C) to the Scottish Centre for Production Animal Health and Food Safety, using a transportation case (Nomad Medical, The Cool Ice Box Company, UK) with hard and soft gel packs. Samples (n = 444) were stored frozen until microbiological analysis. The second sample from each quarter, also collected on the first day of lactation, was collected into pre-labelled containers with milk preservative (0.005 ml of bronopol; 2-bromo-2-nitropropane-1,3-diol) (Fig 5-9B) provided by the National Milk Laboratory (NML) and immediately refrigerated at 4°C for subsequent SCC analysis (n = 436). Day 1 milk samples (colostrum) were. To avoid errors in sample collection, e.g. incorrect matching of containers with quarters, or aseptic collection of samples for culture, distinct vials and bespoke colour coding were used.

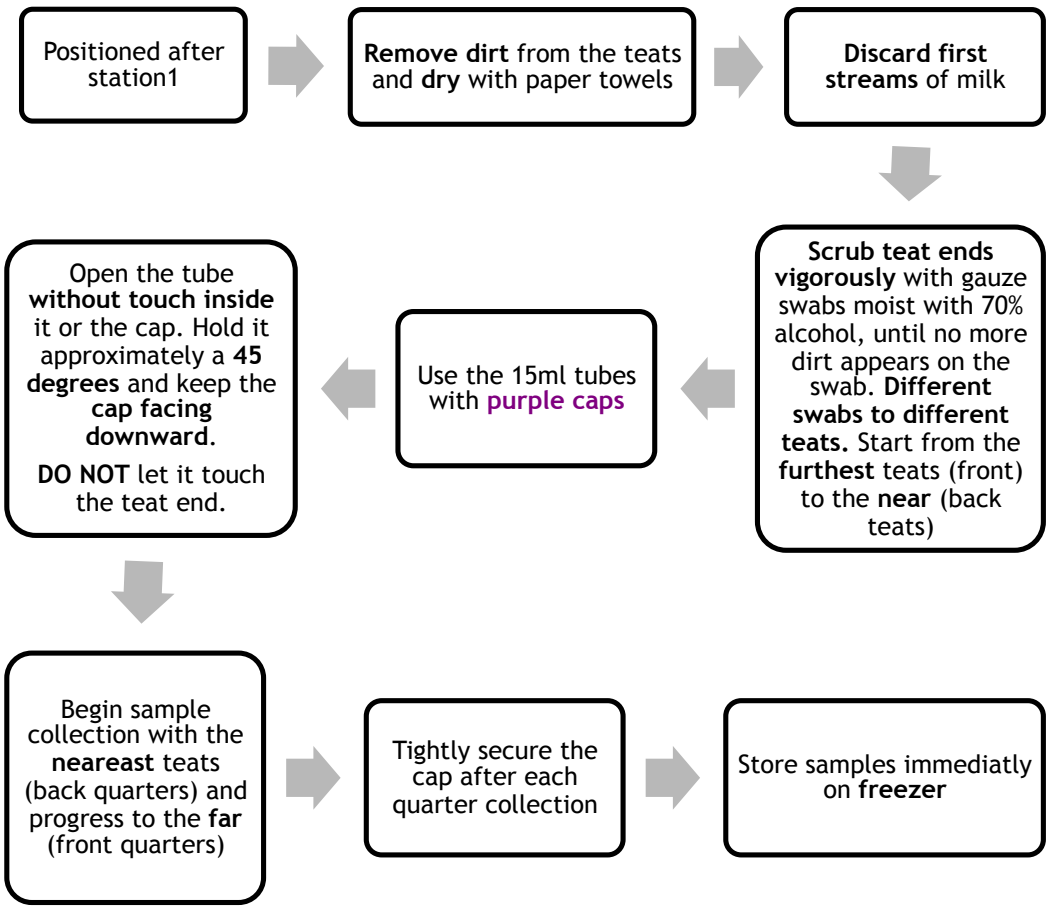

Figure 5-8 Aseptical milk sampling procedure tailored to on-farm use in the rotary parlour.

From the subset of heifers that calved over the first ten weeks of 2015 (n = 24), an additional milk sample was collected into 50 ml sterile centrifuge tubes (VWR international Ltd, UK) (Fig 5-9C) on the first, third and fifth days of lactation. Each of those samples were split after collection, with approximately 20 ml transferred to pre-labelled containers with milk preservative supplied by the National Milk Laboratories (NMR Co., Hillington Park, Glasgow) and kept refrigerated at 4°C for SCC measurements. The remainder of each sample was frozen immediately at -20°C in the original 50 ml tube for haptoglobin measurement. The samples obtained from this subset were used for a pilot study into milk haptoglobin as an indicator of udder health in dairy heifers, with details described Appendix 2.

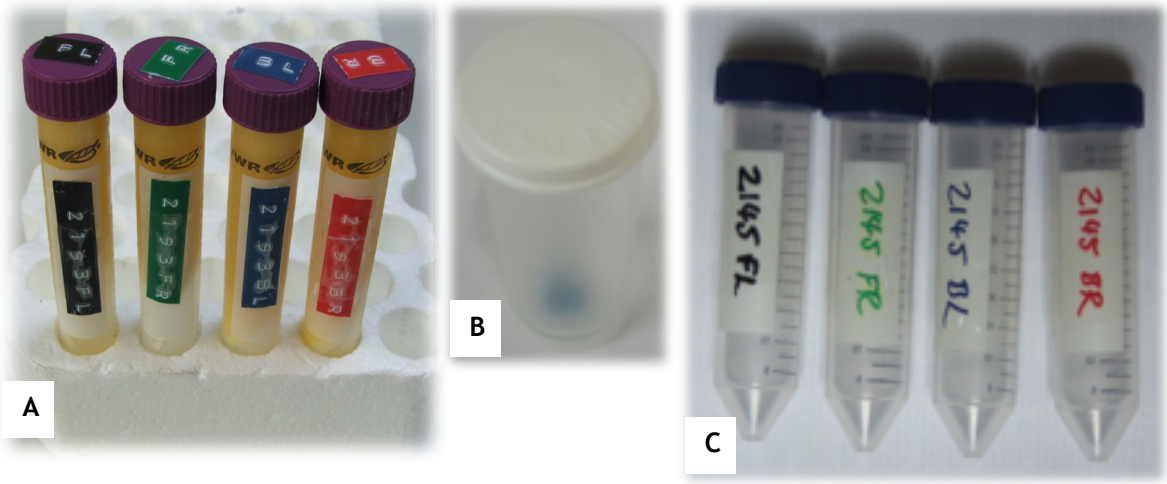

Figure 5-9 Containers used in collection of milk samples for different purposes: A - Microbiology; B - Somatic Cell Count; C - Haptoglobin (Hpt). Vials for microbiology and Hpt measurement were pre-labelled with heifer identification numbers and colour coded by udder quarter to avoid mismatches between quarters and sampling containers (number - animal's last 4 eartag digits; FL - Front Left quarter; FR - Front Right quarter; BL - Back Left quarter; BR - Back Right quarter).

All animals in the herd that presented with CM during lactation were sampled for culture following the routine mastitis protocol in place on the farm.

5.3.5 Laboratory work

5.3.5.1 Somatic cell count

SCC was measured using flow cytometry somatic cell counter technology (Fossomatic, Foss, Hillerød, Denmark) at the National Milk Laboratory (NMR Co., Hillington Park, Glasgow) within four days of sample collection.

5.3.5.2 Milk microbiology

Bacteriological cultures were carried out by the author within 3 weeks of sample collection. The samples were thawed under refrigeration for approximately 15 hours, warmed to room temperature and mixed thoroughly. Then, 0.01 ml of each quarter colostrum sample was inoculated onto a quadrant of a Sheep Blood Agar 5% plate (E&O Laboratories Limited, Bonnybridge, Scotland), using 10 μl calibrated sterile plastic loops (Fig 5-10A). All plates were incubated aerobically up to 48 hours at 37°C. Because there was no history or suspicion of *Mycoplasma* infection on the farm, use of bespoke media for *Mycoplasma* was not considered necessary.

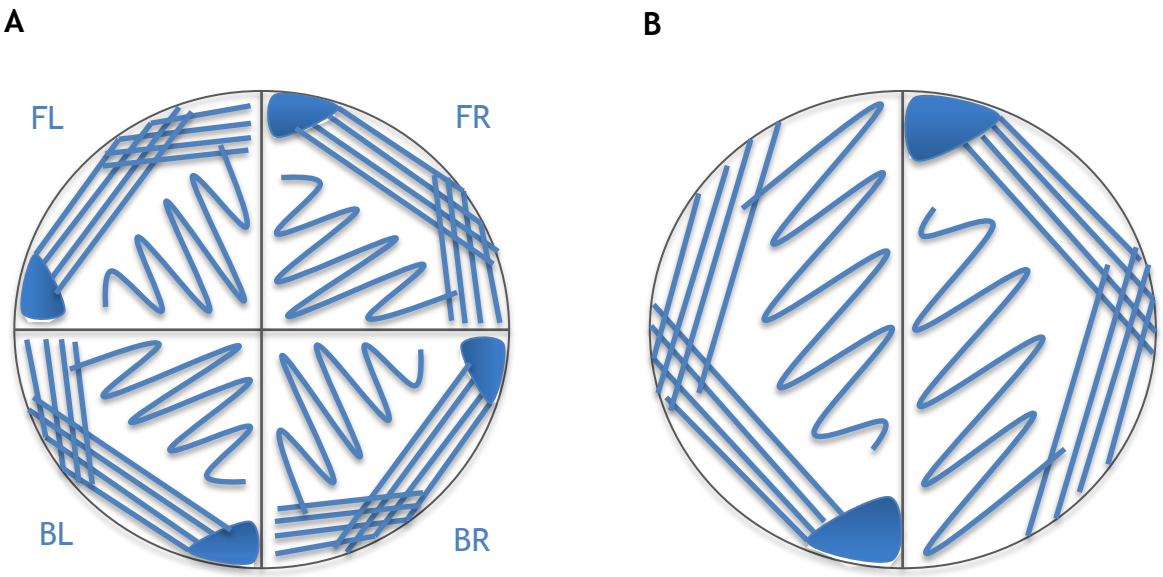

Figure 5-10 Diagram of streaking method used for microbiological culture: A - Initial culture on a plate divided in quadrants; B - Sub-cultures on a plate divided in halves; FL - Front Left quarter; FR - Front Right quarter; BL - Back Left quarter; BR - Back Right quarter.

The number of colonies (colony-forming units – CFUs) of each morphotype was counted at 24 hours (maximum 26 hours) and 48 hours (maximum 50 hours) of

incubation, with recourse to a magnifier and light when necessary. A standard picture was taken from each plate and colony counts recorded. A quarter was considered infected when ≥ 1000 CFU/ml (≥ 10 CFU/10 μl) of a bacterial morphotype was present (Andersen *et al.*, 2010). Samples that contained three or more bacterial morphotypes were considered contaminated, and not used for bacterial isolation or to determine IMI status (Dohoo *et al.*, 2011).

For infected quarters, one colony of each morphotype was picked and re-plated on a half plate of Sheep Blood Agar 5% (Fig 5-10B) and incubated aerobically for 24 hours at 37°C to obtain isolates in pure culture. After incubation, one colony was transferred to 2 ml brain heart infusion broth (BHI) (Oxoid Ltd., UK) and incubated for 24 hours at 37°C. A negative control (uninoculated BHI broth) was used to check for possible contamination (Fig 5-11). After incubation, 850 μl of the overnight culture was transferred to cryovials of 2 ml and combined with 150 μl of glycerol. The cryovials were kept frozen at -80°C for long-term storage and subsequent species identification. The remaining overnight culture was used to distinguish between gram-positive and gram-negative bacteria, using standard Gram staining method.

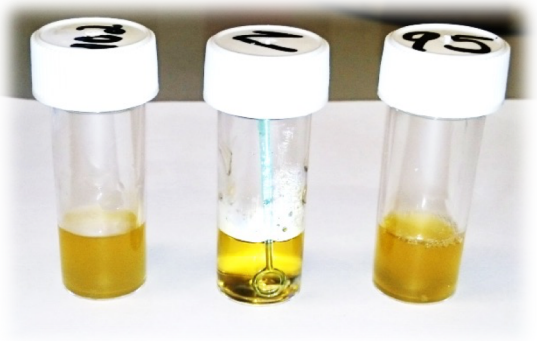

Figure 5-11 Inoculated brain and heart infusion broth after overnight incubation, with negative control (sterile broth sham-inoculated with sterile loop) in the centre. Left and right bijous cloudy due to the presence of bacterial species.

Due to a problem with the freezer, at the end of October 2015, all stored isolates had to be re-plated, incubated in BHI and stored again at -80°C, following the procedures described above. Later, isolates were inoculated on 5% Sheep Blood Agar, incubated and transferred to transport swabs (Transwab

Amies Charcoal Transport, Medical Wire & Equipment, Corsham, UK), that were sent to a collaborator at University College Dublin – School of Veterinary Medicine, Dr James Gibbons, for identification of bacterial species.

After culture of the bacterial isolates present on the transport swabs, Gram stains, oxidase tests (Oxoid Ltd., UK) and catalase tests were carried out on all isolates. Initially, *Staphylococcus aureus* was differentiated from other staphylococcal species based on Pastorex Staph Plus test (BioRad, Watford, UK) and slide coagulase testing, which yielded poorly consistent and poorly reproducible results. Subsequently, the tube coagulase test with rabbit plasma was carried out to differentiate coagulase positive and coagulase negative isolates, with the former presumed to be *Staph. aureus*. Differentiation between streptococci and enterococci was based on the absence of growth on MacConkey agar. To identify streptococci to Lancefield group level a Streptococcal Grouping Kit (Oxoid Ltd., UK) was used assuming that streptococci reacting with antisera to group B were *Strep. agalactiae* and that those reacting with group C were *Strep. dysgalactiae*. Streptococci that failed to react with antisera to groups A, B, C, or D and that were positive for aesculin hydrolysis were assumed to be *Strep. uberis*. Enteric gram-negative organisms were identified using the API20E system (bioMérieux UK Ltd., Basingstoke, UK) and identification of non-enteric gram-negative organisms was completed using the API 20NE system (bioMérieux UK Ltd.). Presumptive identification of gram-positive bacilli was carried out to genus level based on colonial characteristics and catalase testing (Barrow and Feltham, 2004). After bacterial identification by Dr. Gibbons, quarters were classified by the author as infected with major (e.g. *Strep. uberis* and *Staph. aureus*) or minor pathogens (e.g. CNS and *Corynebacterium* spp.) based on the results. Identification of bacterial strains with molecular techniques was not conducted as initially planned due to funding constraints.

5.4 Data analysis

Data was gathered and stored in a Microsoft Excel database (Microsoft® Excel 2010, Microsoft Corporation, Redmond, WA) and imported into SPSS Versions 17.0 and 22.0 (SPSS Inc, Chicago, USA) for analysis, as detailed below.

5.4.1 Definitions used for analysis

5.4.1.1 Temperature

The statistical analysis of the IRT image descriptive parameters was focused on the parameter maximum temperature ('max') from the quarter area as defined by the polygons tool, from the teats as obtained by the lines tool and from the teat meatus temperature as measured through the 'spot meter' tool. Results from the polygons were used because they encapsulate temperature data from a greater area of the udder skin, which is considered more representative than temperature data from a line (Metzner *et al.*, 2014). For the teats, however, the lines tool was used because of the shape of the teats. For teats, curvature is large relative to polygon area, which can lead to detection errors in the temperature readings caused by the surface angle (McCafferty, 2007). The parameter minimum temperature ('min') was also considered, but for the study of the relationship of IRT and postpartum udder health indicators 'max' was preferred due to the fact that the increase of temperature ("calor", or heat) is one of the characteristics of inflammatory processes (Hovinen *et al.*, 2008). Air velocity was low because of the indoor location used for image collection and consequently a low heat exchange by convection was assumed (Nääs *et al.*, 2010). Due to the variability of ambient temperature during data collection (Fig 6-5) and to account for possible heat loss by radiation, illustrated under Results (section 6.1.4), the temperature gradient was used for the analysis (McCafferty *et a*l., 2013; Mellish *et al.*, 2015). The temperature gradient (dT) between surface temperature (T_s) and ambient air temperature (T_{amb}) was calculated according to $dT = T_s - T_{amb}$ for each udder part (Nääs *et al.*, 2010; McCafferty *et al.*, 2013; Mellish *et al.*, 2015; Gebremedhin and Wu, 2016).

5.4.1.2 Infection status

Bacteriological culture results read at 24 hours of incubation were considered in the statistical analysis and treated as categorical variable with three levels (culture-negative, <10 CFU per plate or IMI if ≥10 CFU per plate as in Andersen *et al.* (2010) and also with five levels (culture-negative, <10, 10 to 49, 50 to 199 and ≥200 CFU per plate as in Zadoks *et al.* (2003). Bacterial species were treated as a categorical variable with three levels (culture-negative, major and minor pathogen); when two bacterial species were isolated simultaneously, e.g. one from each category in a single sample, those quarters were classified as infected

with a major pathogen because the major pathogen was expected to dominate the inflammatory response.

5.4.1.3 Inflammatory status

For geometric mean calculation of SCC at day 1 (SCC1), data points with a value of 0 were substituted by 0.9 to allow for log transformation. Considering the low detection limit of 1 x1000 cells/ml of Fossomatic cell counting, this value was just below the detection limit. To approximate the normal distribution and for graphical comparison with IRT data, a natural logarithmic transformation of the SCC1 (x1000 cells/ml) was used. When not used as continuous variable, SCC1 was categorized in four levels based on quartiles of LnSCC1 (very low <4.70, low [4.70 - 6.40[, moderate [6.40 - 7.49[and high >7.49, equating to SCC1 values of <110 x1000 cells/ml, [110 x1000 cells/ml – 604 x1000 cells/ml[, [604 x1000 cells/ml - 1781 x1000 cells/ml[and >1781 x1000 cells/ml, respectively).

5.4.1.4 Boxplots

Distributions of SCC1 and IRT temperatures were presented using boxplots. The boxplot splits the data set into quartiles. The box indicates the interquartile range, third quartile (Q3) to first quartile (Q1). Within the box, the thick black horizontal line indicates the median (second quartile). The whiskers (T-bars) go to from the third quartile to the largest non-outlier of the data set and from the first quartile to the smallest non-outlier. Outliers are plotted separately as circles and extreme outliers as asterisks, above or under the whiskers.

5.4.2 Statistical analysis at quarter level

Statistical analyses were conducted with quarter as unit of analysis. Although quarters are obviously clustered within an animal, for example anatomically, genetically, and with regards to exposure to risk factors, they are functionally separated and infection and inflammation status may differ between quarters. To maximize the number of units observed, and in recognition of the separate physiological status of individual quarters, analysis at quarter level was considered more relevant than analysis at animal level, as in previous mastitis studies (Zadoks *et al.*, 2001). Histograms, boxplots, Q-Q plots, skewness and kurtosis values, and Kolmogorov-Smirnov tests with Lilliefors significance correction were applied to check data distribution normality, with $p > 0.05$ used

to indicate normality. Homogeneity of variances was checked with Levene's test. Median and interquartile range were preferred to describe IRT and SCC data in favour of mean and standard deviation or standard error of the mean, due to their robustness to outliers and non-normal data distribution.

Pearson product-moment correlation coefficient (*r*) was used at quarter level to determine the relationship between IRT temperatures within different projections, and associations with time of IRT collection and ambient and rectal temperature. The strength of the correlation was considered "very weak" when the absolute *r* value ranged from 0.00 to 0.19, "weak" for 0.20 to 0.39, "moderate" for 0.40 to 0.59, "strong" for 0.60 to 0.79, and "very strong" for values from 0.80 to 1.0 (Evans, 1996). Differences between groups of each variable of postpartum udder health indicator were assessed using Kruskal-Wallis *H* (means rank) test, with subsequent Mann-Whitney *U* test for pairwise comparisons, as a nonparametric statistical procedure. Bonferroni adjustment of *p*-values was used for multiple pairwise comparisons to reduce type I error, where p<p/number of pairwise comparison (Green and Salkind, 2004).

Receiver operating characteristics curves (ROC) were used to assess the performance of each IRT parameter as diagnostic tool to detect quarter IMI, using microbiological culture results as reference method for IMI (Dohoo et al., 2011). The area under the ROC curve (AUROC) values can range between 0 and 1.0 (Gardner and Greiner, 2006), and values close to 0.5 often indicate that the test is not suitable as tool for diagnosis (Swets, 1988; Gardner and Greiner, 2006; Berrar and Flach, 2012). Associations between IRT data and postpartum udder health indicators were analysed using binary logistic regression (quarter status: IMI, No IMI) and ordinal logistic regression though PLUM and GENLIN procedures (LnSCC1 categories). Omnibus test was used as part of the binomial logistic regression procedure to check if the models were an improvement over the baseline model, whereas Likelihood-ratio test was used as part of the ordinal logistic regression to look to changes in model fit when comparing the full model with the intercept-only model. Linearity of the continuous variables with respect to the logit of the dependent variable was assessed via the Box-Tidwell (1962) procedure. Four models based on the time of IRT (2 months or 2 weeks pre-partum) and the two projections were tested for each dependent variable

(quarter status and LnSCC1 categories). Variables included in the caudocranial projection models were $dT_{'max'}$ of quarters and $dT_{'max'}$ of teats, and in the ventrodorsal projection models $dT_{'max'}$ of quarters, $dT_{'max'}$ of teats and $dT_{'spot'}$ of teat orifices, following the model equation:

$\text{logit}(Y) = \beta_0 + \beta_1 \times \text{quarter} + \beta_2 \times dT_{'max'}$ of quarter $+ \beta_3 \times dT_{'max'}$ of teat $+ \varepsilon$, for caudocranial projection;

$\text{logit}(Y) = \beta_0 + \beta_1 \times \text{quarter} + \beta_2 \times dT_{'max'}$ of quarter $+ \beta_3 \times dT_{'max'}$ of teat $+ \beta_4 \times dT_{'spot'}$ of teat orifice $+ \varepsilon$, for ventrodorsal projection;

Where β_0 is the intercept; β_1, β_2 , β_3 and β_4 are the coefficients or slope parameters for quarter (front right, front left, back right and back left) , $dT_{'max'}$ of quarter (front right, front left, back right and back left), $dT_{'max'}$ of teat (front right, front left, back right and back left) and $dT_{'spot'}$ of teat orifice (front right, front left, back right and back left), respectively; and ε represents the error. Statistical significance level was defined at $p \leq 0.05$.

6 Results

6.1 IRT results pre-partum

6.1.1 Environmental conditions

Ambient air temperature (T_{amb}) varied from 1.5°C to 11.9°C (mean ± *SE* = 7.44 ± 0.31°C) at the time of collection of the 1st set of IRT data (2 months pre-partum) and from 3.2°C to 17.6°C (mean 9.03 ± 0.35°C) at the time of collection of the 2nd set of IRT data (2 weeks pre-partum). Relative humidity ranged from 38.9 % to 68.3 % (mean 54.0 ± 0.38 %) and from 24.0 % to 58.2 % (mean 44.2 ± 0.46 %) at the time of the first and second IRT image collection, respectively.

6.1.2 Core and surface temperatures

From the one hundred and twenty animals enrolled in the study, one hundred and nineteen were subjected to the 1st IRT scan (mean ± *SE* of 63.81 ± 0.53 days pre-partum); the missing animal was present at the 2nd IRT and was also included in subsequent data and sample collection. One hundred and sixteen heifers were submitted to 2nd IRT (mean 16.91 ± 0.26 days pre-partum). Six IRT sessions were conducted for collection of the 1st set of IRT images and 21 sessions for the collection of the 2nd IRT set of images.

6.1.2.1 Core temperature

Body temperature of the heifers based on rectal temperature was 38.82 ± 0.04°C (mean ± *SE*) at the first IRT and 38.59 ± 0.03°C at the second IRT (Fig 6-1).

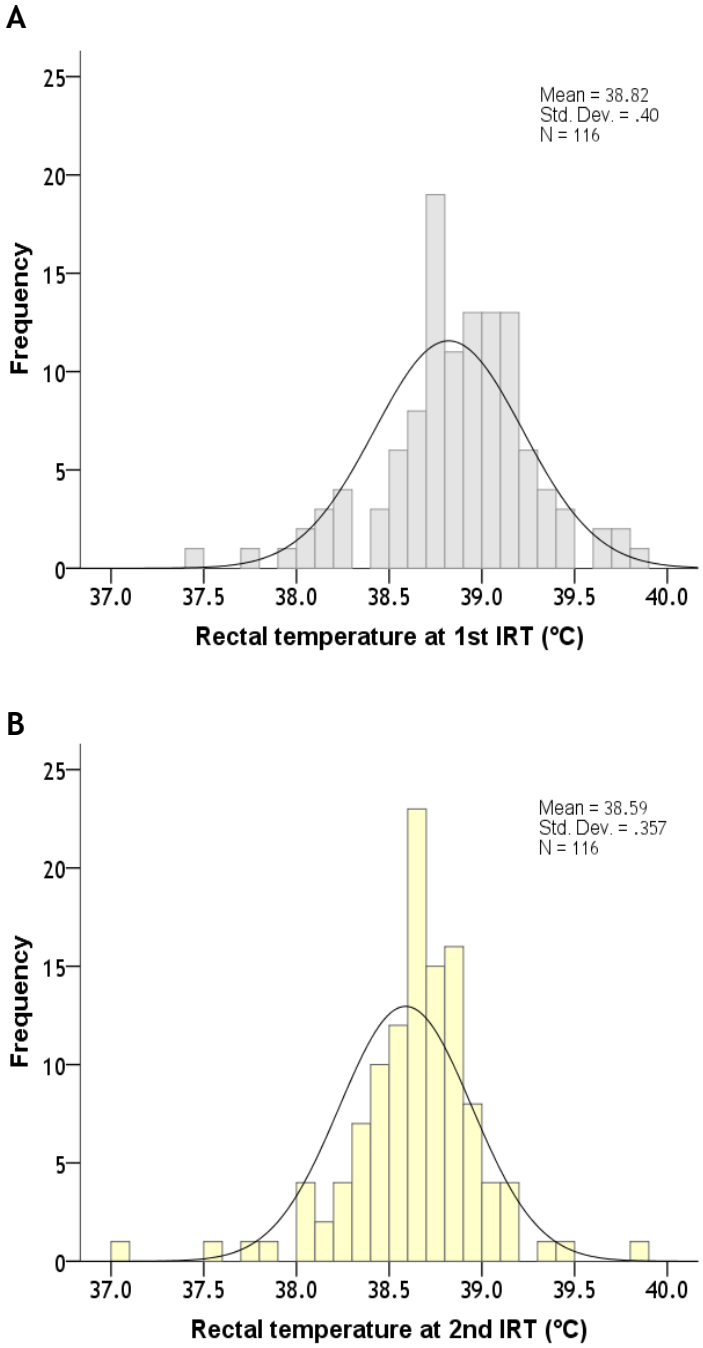

Figure 6-1Distribution of rectal temperatures at time of first IRT (A) and second IRT (B) image collection. Curves show fitted normal distributions.

6.1.2.2 Surface temperature

Body surface temperatures (T_s) and temperature gradients (dT) for quarter, teat, or teat orifice in both projections are presented in Tables 6-1 and 6-2 for IRT images collected at 2 months and 2 weeks pre-partum, respectively.

Table 6-1 Surface (T_s) and temperature gradient (dT) at first IRT (Cd-Cr n= 238, i.e. both rear quarters for 119 heifers; Vt-Dr n= 476, i. e. 4 quarters each for 119 heifers).

			Temperature (°C)					
Udder region	Projection		$T_{s'min'}$	$T_{s'max'}$	$T_{s'spot'}$	$dT_{'min'}$	$dT_{'max'}$	$dT_{'spot'}$
Quarter	Cd-Cr	*Median*	23.55	34.00		15.70	25.95	
		Q1	20.4	32.5		13.3	24.5	
		Q3	26.0	34.7		17.9	28.0	
	Vt-Dr	*Median*	25.25	34.50		17.00	26.20	
		Q1	22.2	33.5		14.5	24.7	
		Q3	27.8	35.2		19.9	29.1	
Teat	Cd-Cr	*Median*	23.20	29.30		15.10	21.40	
		Q1	19.2	27.1		12.2	19.8	
		Q3	26.4	31.5		18.0	23.1	
	Vt-Dr	*Median*	24.05	31.50		16.40	23.40	
		Q1	19.9	29.8		12.8	22.2	
		Q3	27.5	33.0		18.7	25.5	
Teat orifice	Vt-Dr	*Median*			23.05			15.45
		Q1			19.2			11.8
		Q3			26.4			18.0

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; Q1- first quartile and Q3- third quartile: Tukey's Hinges; 'min' – minimum; 'max' – maximum; 'spot' – point temperature.

Table 6-2 Surface (T_s) and temperature gradient (dT) at second IRT (Cd-Cr n= 232, i.e. both rear quarters for 116 heifers; Vt-Dr n= 464, i.e. 4 quarters each for 116 heifers).

			Temperature (°C)					
Udder region	Projection		$T_{s'min'}$	$T_{s'max'}$	$T_{s'spot'}$	$dT_{'min'}$	$dT_{'max'}$	$dT_{'spot'}$
Quarter	Cd-Cr	*Median*	25.95	34.40		16.70	25.75	
		Q1	23.6	33.5		14.4	22.0	
		Q3	28.2	34.9		18.8	27.5	
	Vt-Dr	*Median*	28.10	36.8		18.50	26.70	
		Q1	25.8	34.4		15.7	23.0	
		Q3	29.7	35.6		21.2	28.5	
Teat	Cd-Cr	*Median*	28.80	32.10		19.25	22.80	
		Q1	26.9	30.8		15.6	19.2	
		Q3	30.1	33.0		21.6	25.5	
	Vt-Dr	*Median*	29.30	33.10		19.70	23.75	
		Q1	27.8	30.7		16.3	20.7	
		Q3	31.9	33.8		22.5	26.3	
Teat orifice	Vt-Dr	*Median*			28.50			18.45
		Q1			26.9			15.3
		Q3			30.0			21.9

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; Q1- first quartile and Q3- third quartile: Tukey's Hinges; 'min' – minimum; 'max' – maximum; 'spot' – point temperature.

6.1.3 Association between core and surface temperature

Five animals presented with rectal temperature higher than 39.5°C at 1st IRT. At the time of 2nd IRT, one animal presented with rectal temperature higher than 39.5°C. Maximum surface temperature was positively correlated with rectal temperature at 1st IRT whereas maximum temperature gradients were negatively correlated with rectal temperature at 1st IRT (Table 6-3). At the 2nd IRT measurement the maximum temperature gradients were correlated with rectal temperature, however in contrast to the 1st IRT results it was a positive correlation. These correlations vary from very weak to moderate (Table 6-3).

Table 6-3 Association between rectal temperature (RT) and teat/quarter maximum surface (T_s) and gradient (dT) temperatures at first (Cd-Cr n= 238; Vt-Dr n=476) and second IRT (Cd-Cr n= 234; Vt-Dr n= 464), expressed as Pearson's correlation coefficient (*r*) and coefficient of determination (r^2) .

		Caudocranial projection				Ventrodoral projection			
		Teat		Quarter		Teat		Quarter	
		$T_{s'max'}$	$dT_{s'max'}$	$T_{s'max'}$	$dT_{s'max'}$	$T_{s'max'}$	$dT_{s'max'}$	$T_{s'max'}$	$dT_{s'max'}$
1st IRT	*r*	.22**	-.32***	.43***	-.23***	.12*	-.45***	.21***	-.42***
	r^2	.04	.10	.18	.05	.01	.20	.04	.18
2nd IRT	*r*	-.05	.23***	-.07	.26***	-.001	.26***	.07	.29***
	r^2	.003	.05	.005	.07	.000001	.07	.005	.08

'max' – maximum; 'min' – minimum ***p=.0005, **p= 0.001, *p< 0.05

6.1.4 Association between animal temperatures and ambient temperature

Core temperature, body surface temperatures and ambient temperature were plotted against the date of IRT collection, whereby the median values for $T_{s'max'}$ and $T_{s'min'}$ were used as indicators of body surface temperature. Body surface temperature and ambient temperature followed similar patterns, particularly for the 1st set of IRT data, whilst this pattern was not obvious for core temperature (Fig 6-2). The difference between surface and core temperatures in this regard is compatible with the weak correlation between rectal and surface temperature (Table 6-3). However, both rectal temperature and core temperature were significantly correlated with ambient temperature (Table 6-4). The correlation of core temperature with ambient temperature was positive at 1st IRT and

negative at 2nd IRT, as also observed for the correlation between core temperature and surface temperature.

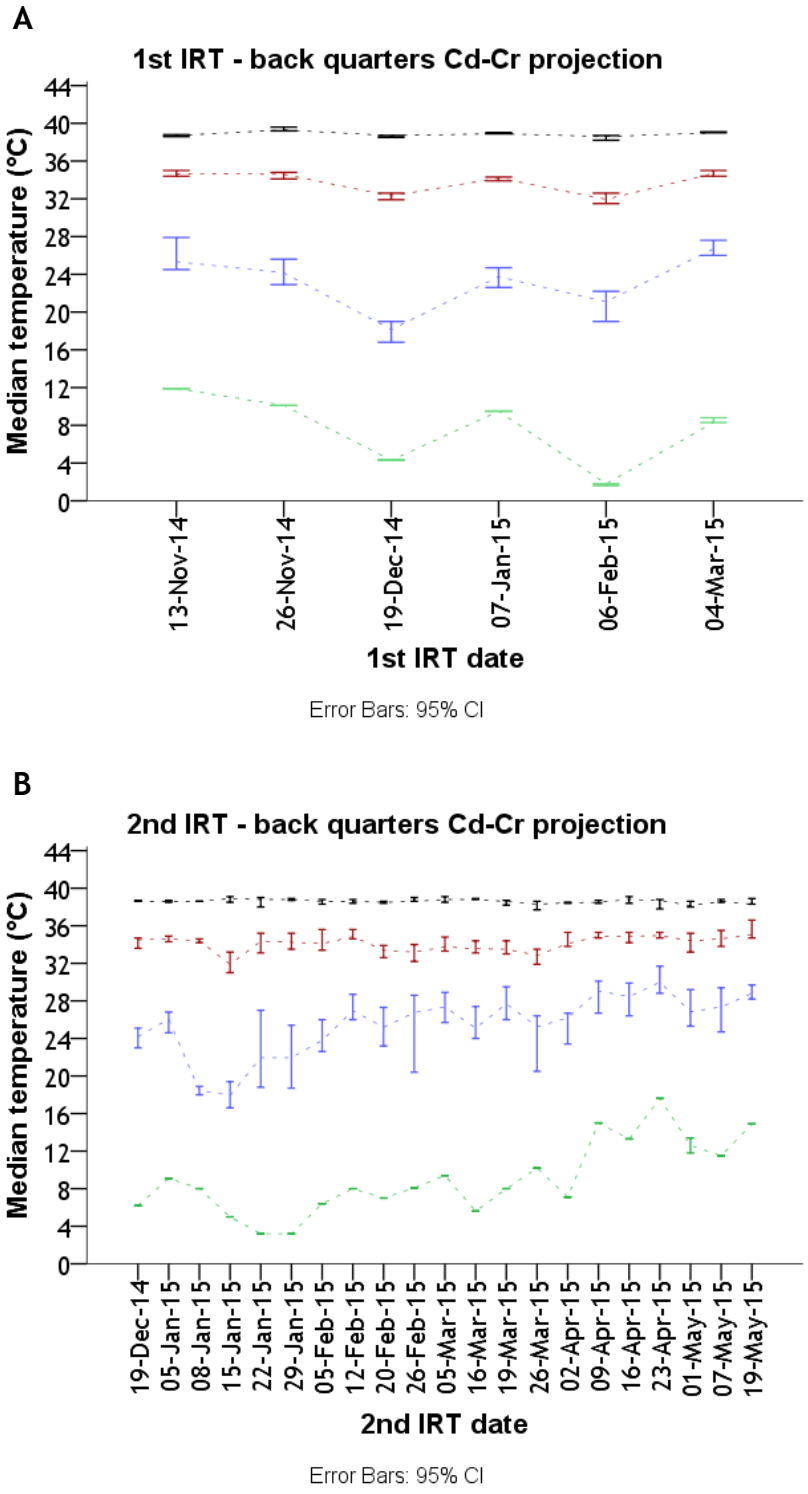

Figure 6-2 Temperature trend by date of IRT collection: A - first IRT; B - second IRT. Rectal temperature (- black bars); quarter $T_{s'max'}$ temperature (- red bars); quarter $T_{s'min'}$ temperature (- blue bars); ambient air temperature (- green bars). Bars indicate the range. Dashed lines are visual guides to help pattern recognition, they should not be interpreted as measurements.

Table 6-4 Pearson's correlation coefficient (r) and coefficient of determination (r^2) between rectal temperature (RT) and quarter surface temperatures (T_s) with ambient air temperature (T_{amb}).

	1st IRT					2nd IRT				
		Cd-Cr		Vt-Dr			Cd-Cr		Vt-Dr	
	RT	$T_{s'max'}$	$T_{s'min'}$	$T_{s'max'}$	$T_{s'min'}$	RT	$T_{s'max'}$	$T_{s'min'}$	$T_{s'max'}$	$T_{s'min'}$
n	119	238	238	476	476	116	234	234	464	464
r	0.43*	0.72*	0.57*	0.58*	0.40*	-0.26*	0.35*	.64*	0.32*	0.56*
r^2	0.19	0.52	0.32	0.34	0.16	0.07	0.12	0.41	0.10	0.31

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; n – number; 'max' – maximum; 'min' – minimum
*p = 0.0005.

6.1.5 Comparison between projections for surface temperatures

A Pearson's product-moment correlation was run to assess the relationship between caudocranial and ventrodorsal IRT projections at the first and second time of IRT image collection. The statistical test was applied to quarters and teats 'max' and 'min' temperature gradients ($dT_{'max'}$ and $dT_{'min'}$). Preliminary analysis showed the relationship to be linear with temperature variables non-normally distributed. Outliers were considered genuine data points, and therefore kept in the analysis. There was a "strong" to "very strong" positive correlation between $dT_{'max'}$ and $dT_{'min'}$ of the caudocranial projection and the ventrodorsal projection of quarters and teats on both IRT occasions, as summarized in the scatterplots (Fig 6-3 and Fig 6-4).

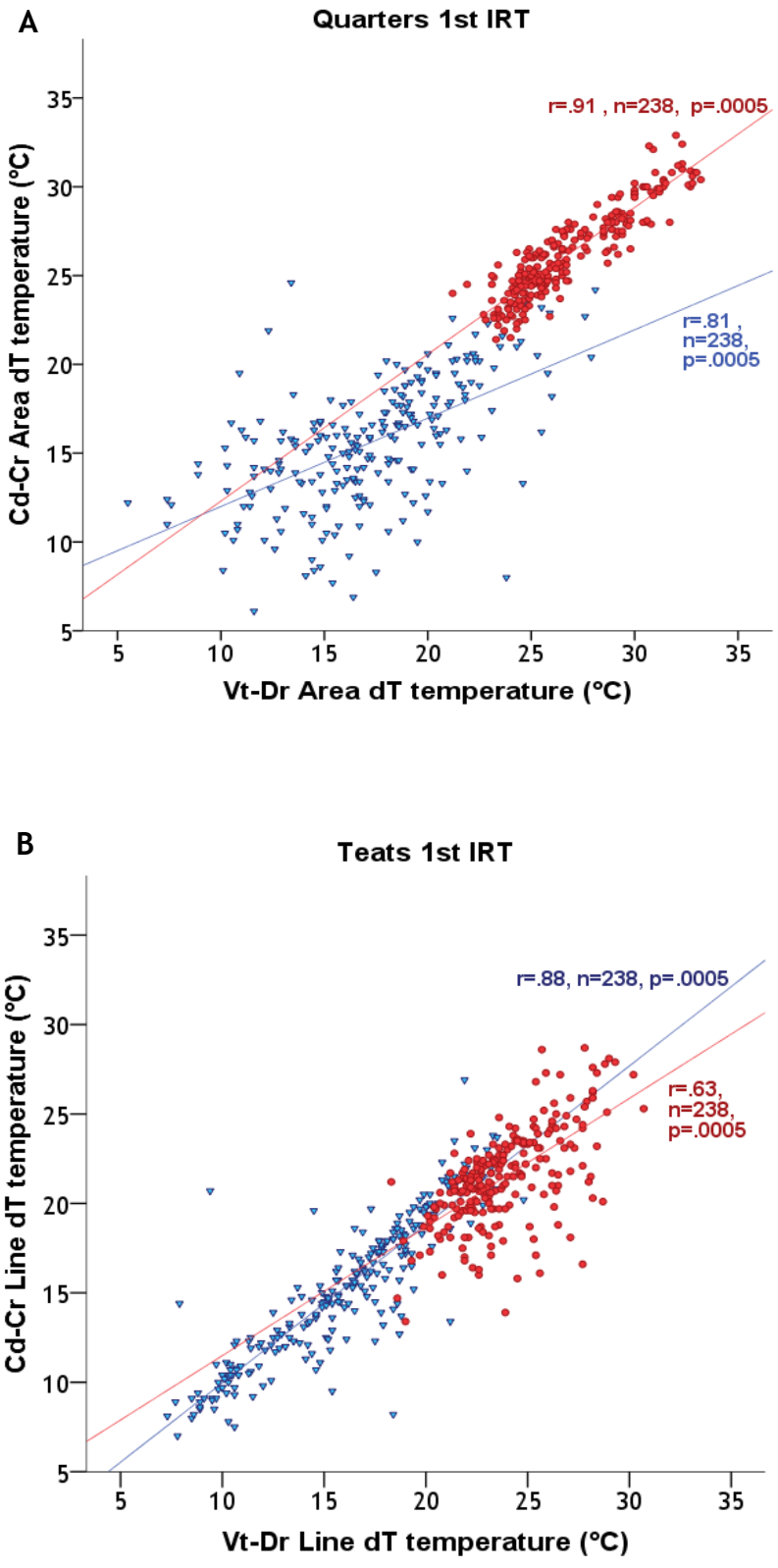

Figure 6-3 Scatterplots summarizing Pearson's correlation (*r*) results between IRT projections at first IRT image collection. Cd-Cr - Caudocranial projection; Vt-Dr - Ventrodorsal projection; dT - temperature gradient; ▼- 'min' temperature; ● - 'max' temperature.

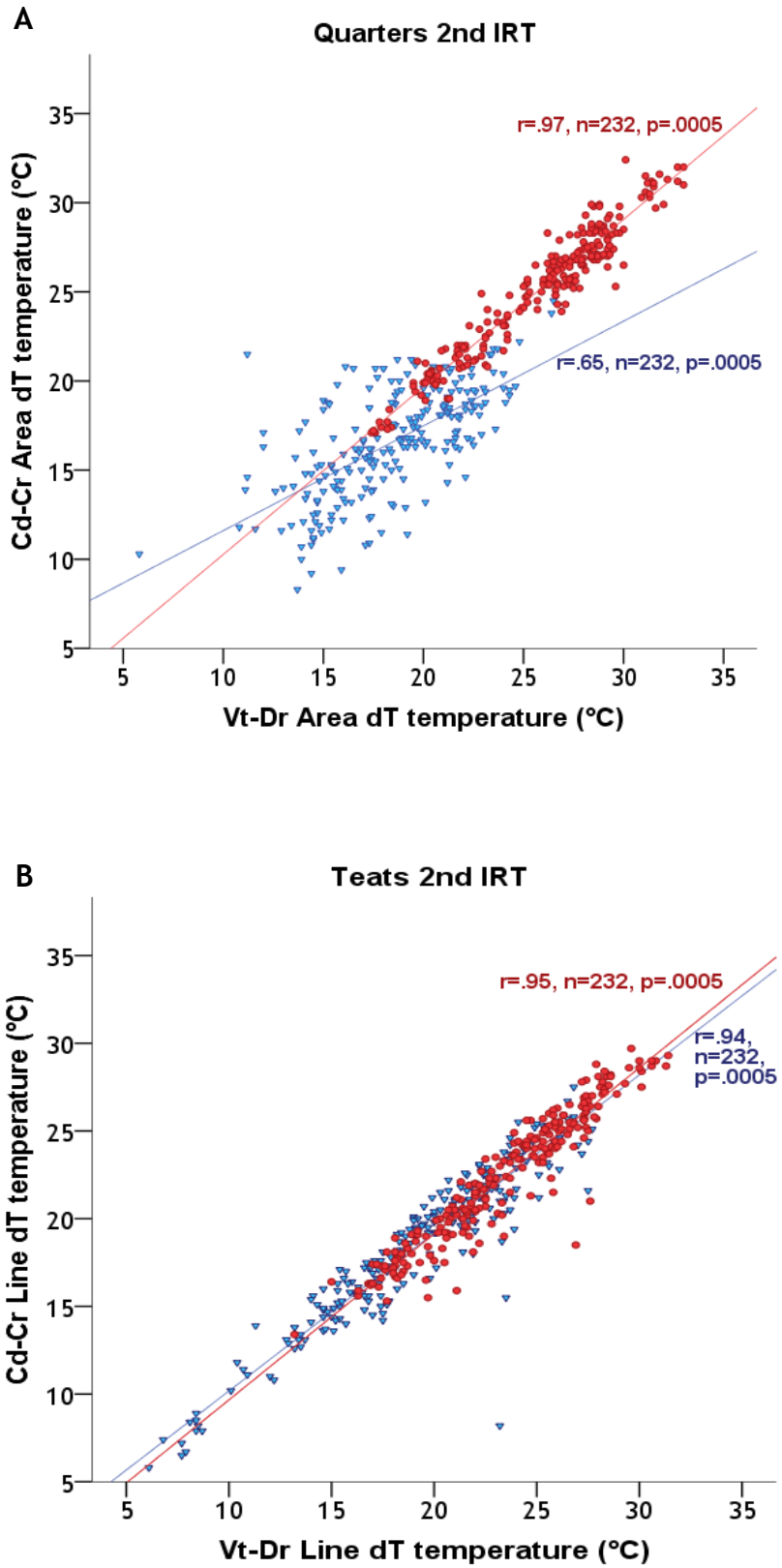

Figure 6-4 Scatterplots summarizing Pearson's correlation (*r*) results between IRT projections at second IRT image collection. Cd-Cr – Caudocranial projection; Vt-Dr – Ventrodorsal projection; dT – temperature gradient; ▼- 'min' temperature; ● - 'max' temperature.

6.2 Udder health indicators post-partum

6.2.1 Microbiology results

6.2.1.1 Quarter-level bacteriology at 1st milking

Of 440 quarter-milk samples cultured, 160 (36%) were culture-negative, 171 (39%) were culture-positive with less than 1000 CFU/ml (<10 CFU per plate), 86 (20%) were considered to have IMI with more than 1000 CFU/ml (≥10 CFU per plate) of a bacterial morphotype, and 23 (5%) were classified as contaminated (Fig 6-5). Quarters with mild growth (n = 39; 10 to 49 CFU per plate) or moderate growth (n = 35; 50 to 199 CFU per plate) were most common, whilst profuse growth (n = 12; ≥200 CFU per plate) was observed on 14% of plates (Fig 6-5).

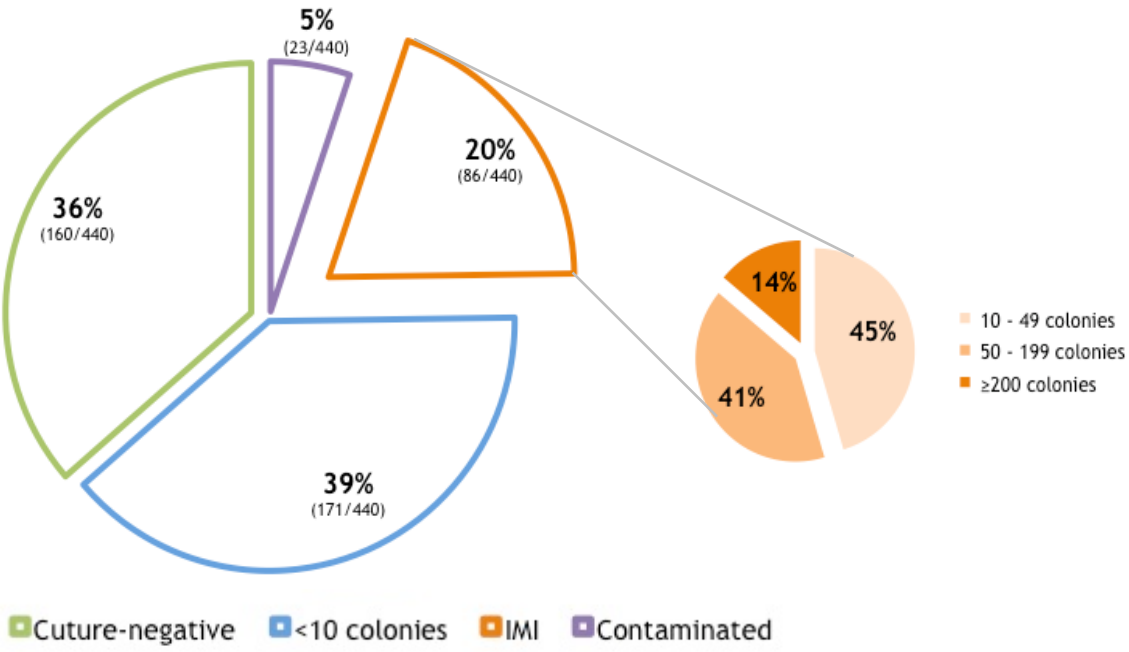

Figure 6-5 Distribution of culture results based on growth, with number of colonies used as indicator of infection status.

6.2.1.2 Bacteria isolated at 1st milking

The colonies from one hundred and thirty-one quarters (all quarters classified with IMI and 45 first quarters sampled during the study with <10 CFU per plate) were identified to genus or species level. The majority of bacteria were classified as minor pathogen, mostly CNS. The major pathogens *Strep. uberis*, *Strep. dysgalactiae* and *Staph. aureus* were also isolated, albeit infrequently.

Quarters that were classified as infected (n = 86) had a slightly lower percentage of CNS positive samples than culture-positive quarters with <10 CFU per plate (n = 45), i.e. 89.7% and 93.4% (Fig 6-6). The percentage of major pathogens isolated was numerically higher in infected quarters (10.7%, Fig 6-6B) than quarters with low CFU count (2.2%, Fig 6-6A but the difference was not significant (Chi-square = 2.58, df = 1, p = 0.09).

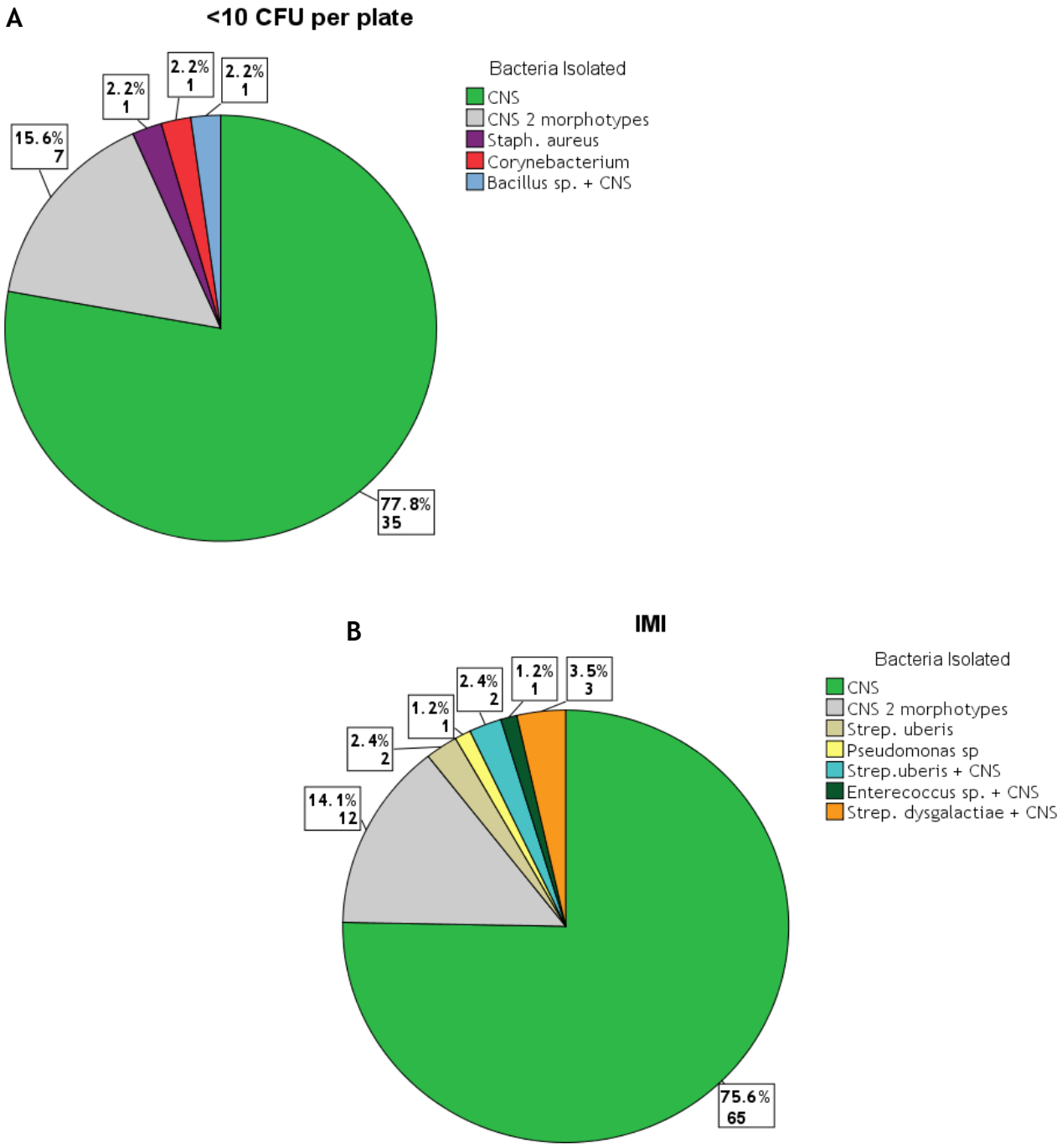

Figure 6-6 Proportion of bacteria identified from quarter-level colostrum samples. A - Culture positive with <10 colony forming units (CFU) per plate (n=45); B - Culture positive and considered indicative of intramammary infection (≥10 CFU per plate) (n=86). CNS = coagulase negative staphylococci.

6.2.2 Somatic cell count results

6.2.2.1 Quarter-level SCC at 1st milking (SCC1)

Median SCC across 436 colostrum samples was 605 x1000 cells/ml, with interquartile range (Q1-Q3) of 1686 (111 – 1782) x1000 cells/ml and geometric mean 419 x1000 cells/ml. SCC1 did not differ significantly between quarter positions ($H(3) = 0.92$ $p = 0.82$), with the back left quarter samples presenting a geometric mean of 395 x1000 cells/ml, back right quarter 408 x1000 cells/ml, front left 442 x1000 cells/ml and front right 432 x1000 cells/ml.

6.2.2.2 Association of SCC1 with bacterial load

For 409 quarter-level colostrum samples, SCC1 data and microbiology data were available, including 158 for culture negative samples, 168 culture positive samples with low cfu count, and 83 samples from quarters with IMI. SCC1 was higher for samples from quarters with IMI than for culture-negative samples or those with <10 CFU per plate (Table 6-5; Figure 6-7; $H(2) = 10.61$, $p = 0.005$ for overall differences in SCC1; $p = 0.008$ for IMI vs. low CFU groups and $p = 0.002$ for IMI vs culture negative group). SCC1 did not differ significantly between culture-negative samples and those yielding <10 CFU per plate ($p = 0.426$), in agreement with the notion that the latter are not necessarily indicative of IMI.

Table 6-5 Summary of SCC1 descriptive statistics by culture status group.

Culture Status	Geometric Mean[a]	Median[a]	Interquartile range[a]
Culture-negative	336	506	89 – 1422[‡]
<10 CFU per plate	365	590	125 – 1652.50[‡]
IMI	867	1103	226 – 4784.50[‡]

[a]x1000 cells/ml; [‡] (first quartile – third quartile) Tukey's Hinges

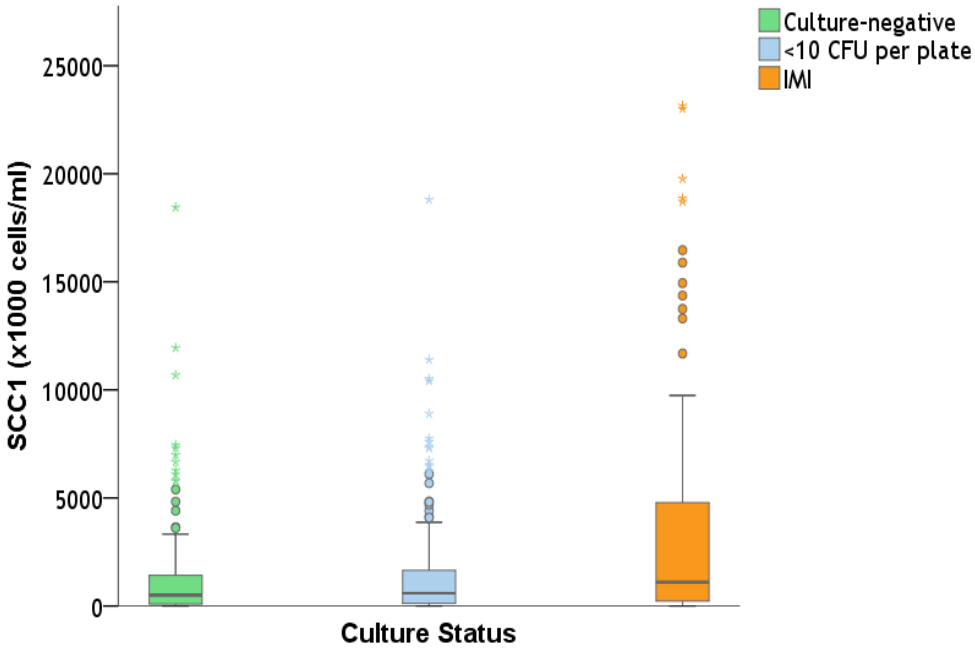

Figure 6-7 Boxplots comparing the distribution of SCC1 in culture status groups. CFU – colony-forming units; IMI – intramammary infection; SCC1 – somatic cell count at calving.

Within the IMI group, geometric mean SCC1 increased numerically but not significantly with the number of CFU counted per plate (Fig 6-8; $H(2) = 3.70$, $p = 0.16$).

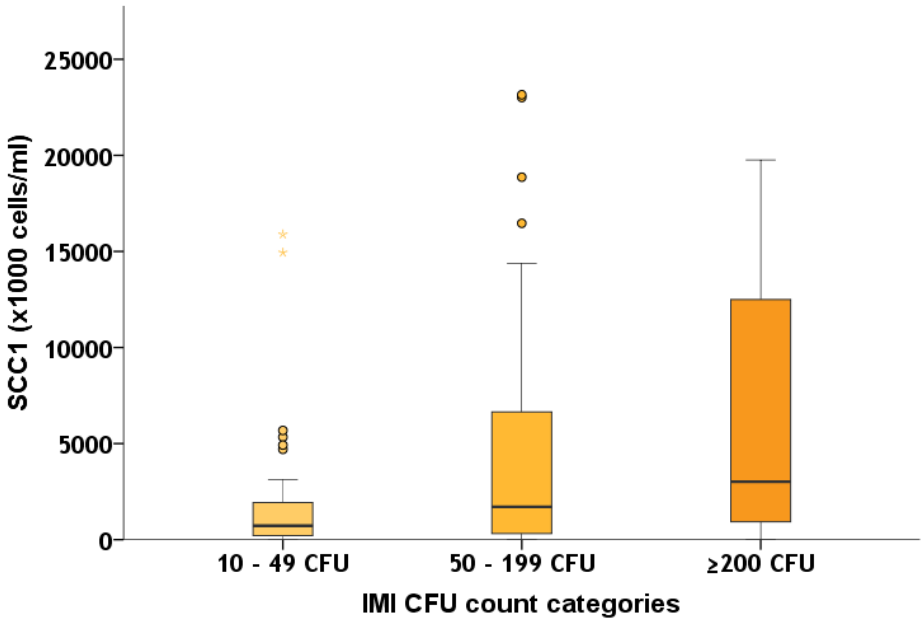

Figure 6-8 Association of somatic cell count at calving (SCC1) with bacterial load in colostrum samples from quarters with intramammary infection (IMI). CFU = colony-forming units.

6.2.2.3 Association of SCC1 with major and minor pathogens

Overall, there was a significant difference between SCC1 for samples yielding no growth, minor pathogens, or major pathogens ($H(2) = 9.97$, $p = 0.007$; Fig 6-9). Pairwise comparison showed that the difference between samples with no growth and those with minor pathogens was significant ($p = 0.002$), while SCC in samples with major pathogens was not significantly different from the other groups, despite obvious differences in median values (Fig 6-9). This is quite possibly due to the limited number of observations, with only 7 SCC observations from quarters with major pathogens, compared with 76 from quarters with minor pathogens and 158 from culture negative quarters.

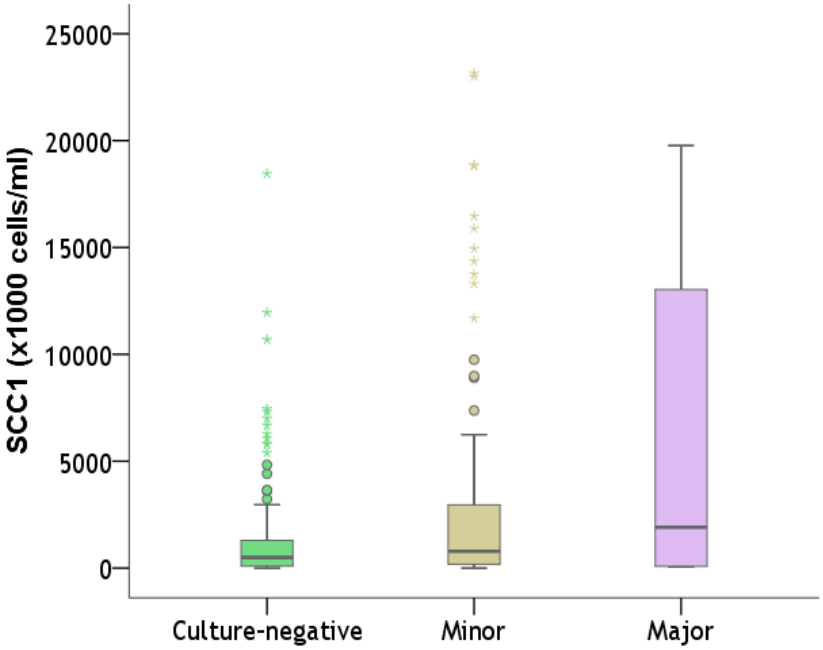

Figure 6-9 Association of SCC1 with culture status. SCC1 - somatic cell count at calving, minor = minor pathogen, major = major pathogen.

6.2.3 Association between IRT and udder health indicators

6.2.3.1 IRT temperature by bacteriological status

Surface temperatures from caudocranial and ventrodorsal projections at 1st or 2nd IRT were not associated with udder health as defined by colony counts, whether this was based on quarter temperature (Fig 6-10) or teat temperatures (Fig 6-11), nor with udder health as defined by pathogen category (Figures 6-12

and 6-12 for quarters and teats, respectively). Details of median temperatures, temperature ranges and statistical comparisons can be found in Appendix 3.

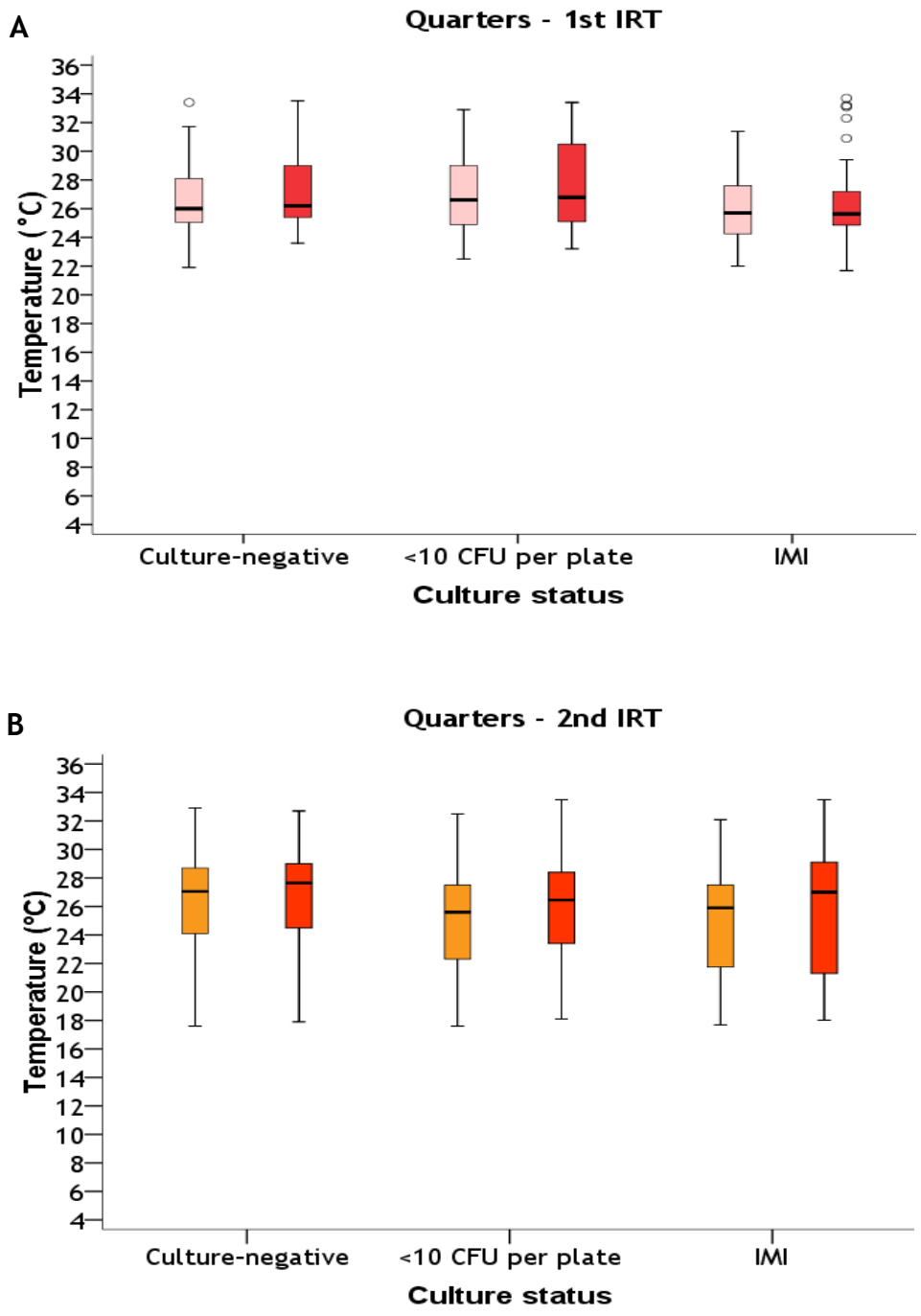

Figure 6-10 Boxplots comparing the distribution of quarter IRT temperature pre-partum by culture status at subsequent calving. A - first IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection. B - second IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection.

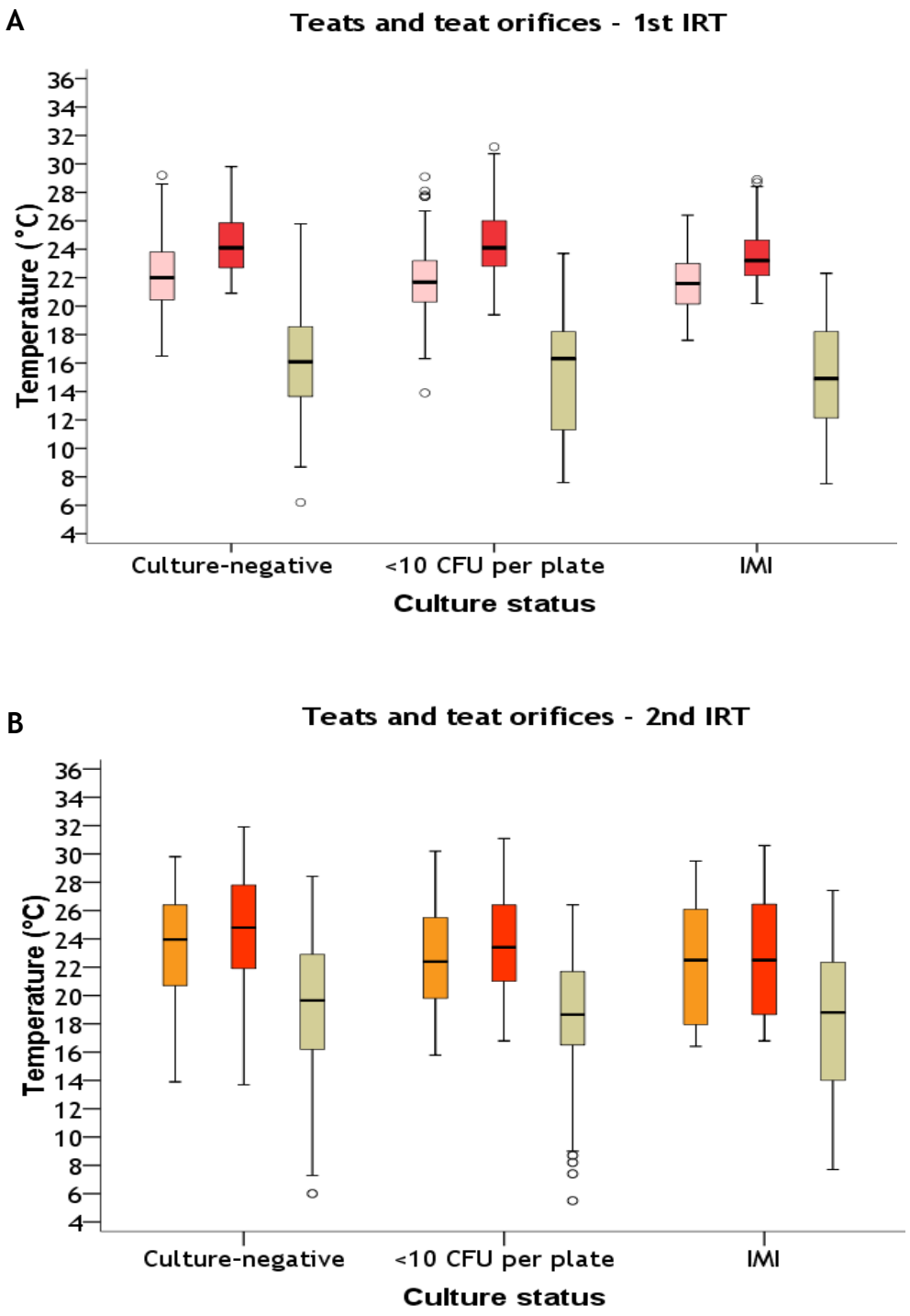

Figure 6-11 Boxplots comparing the distribution of teat and teat orifices IRT temperature pre-partum by culture status at subsequent calving. A - first IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection; ▪ $dT_{'spot'}$ at ventrodorsal projection. B - second IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection; ▪ $dT_{'spot'}$ at ventrodorsal projection.

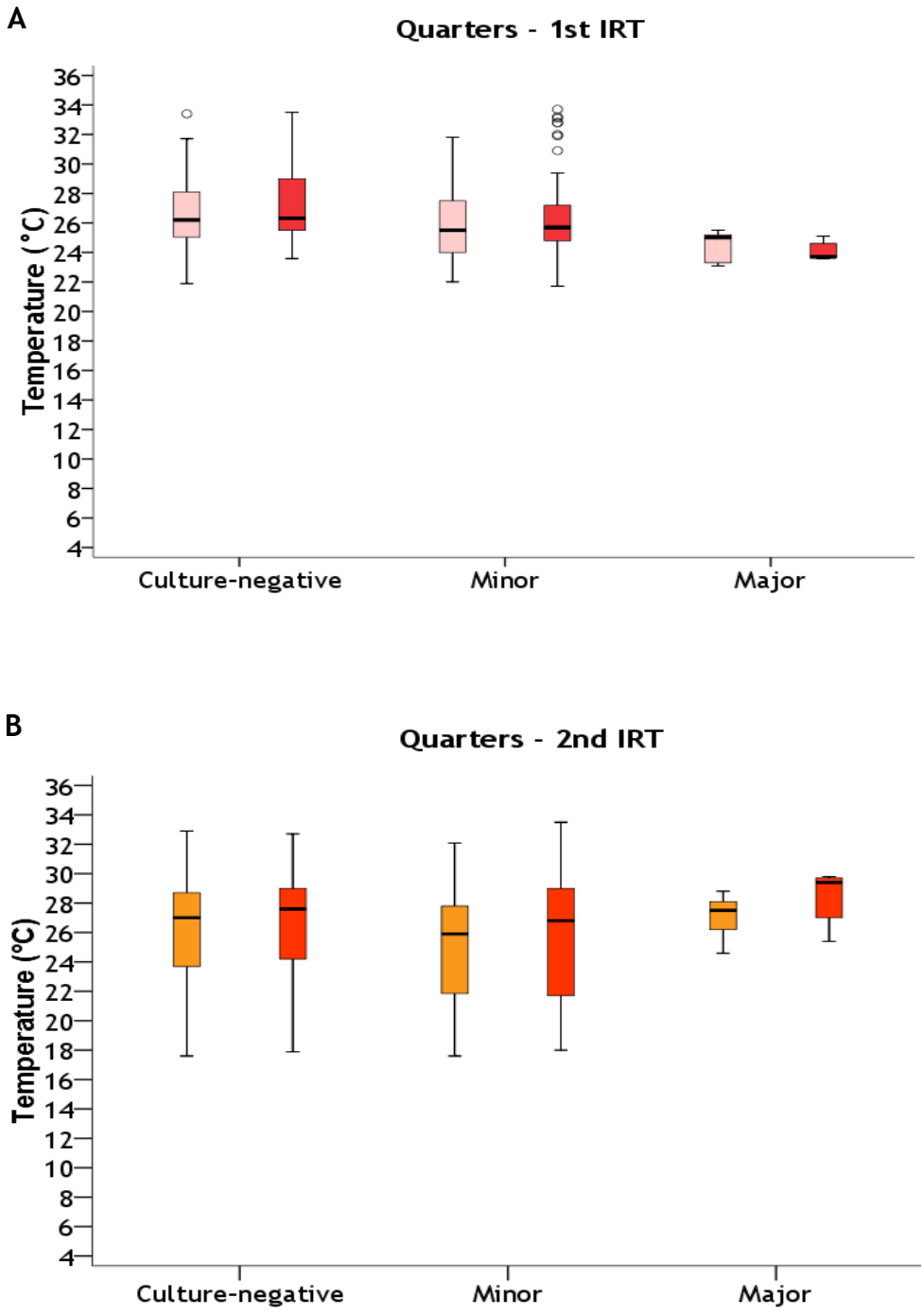

Figure 6-12 Boxplots comparing the distribution of quarter IRT temperature prepartum for quarters that were positive for minor pathogens and major pathogens, respectively, at subsequent calving. A - first IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection. B - second IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection.

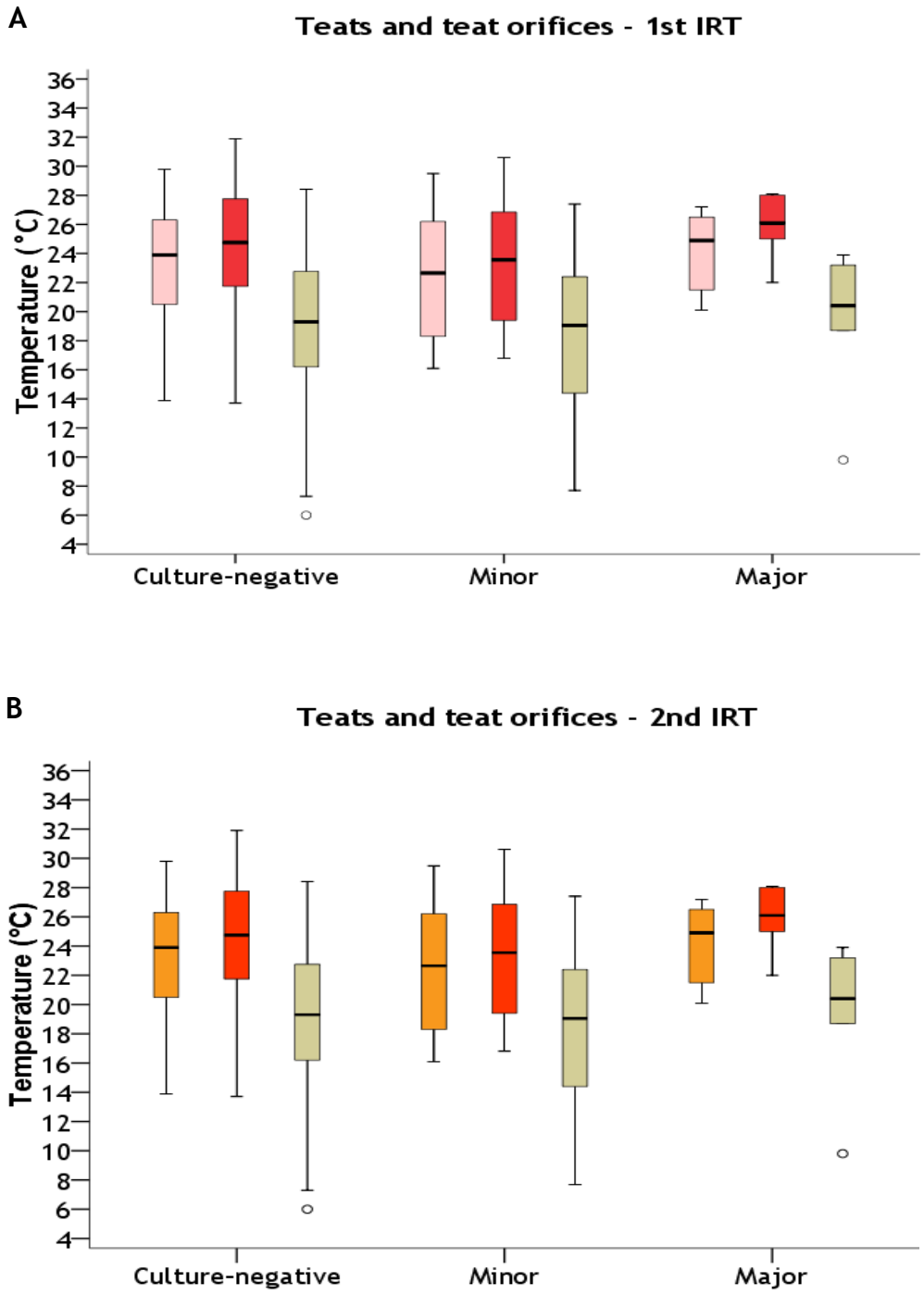

Figure 6-13 Boxplots comparing the distribution of teat and teat orifices IRT temperature pre-partum for quarters testing positive for minor and major pathogens at calving. A - first IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection; ▪ $dT_{'spot'}$ (teat orifice). B - second IRT: ▪ $dT_{'max'}$ at craniocaudal projection; ▪ $dT_{'max'}$ at ventrodorsal projection; ▪ $dT_{'spot'}$ (teat orifice).

From 1st to 2nd IRT, $dT_{'max'}$ increased in most quarters with major pathogens whilst there was a bimodal distribution including temperature increases and decreases in quarters infected with minor pathogens (Fig 6-13).

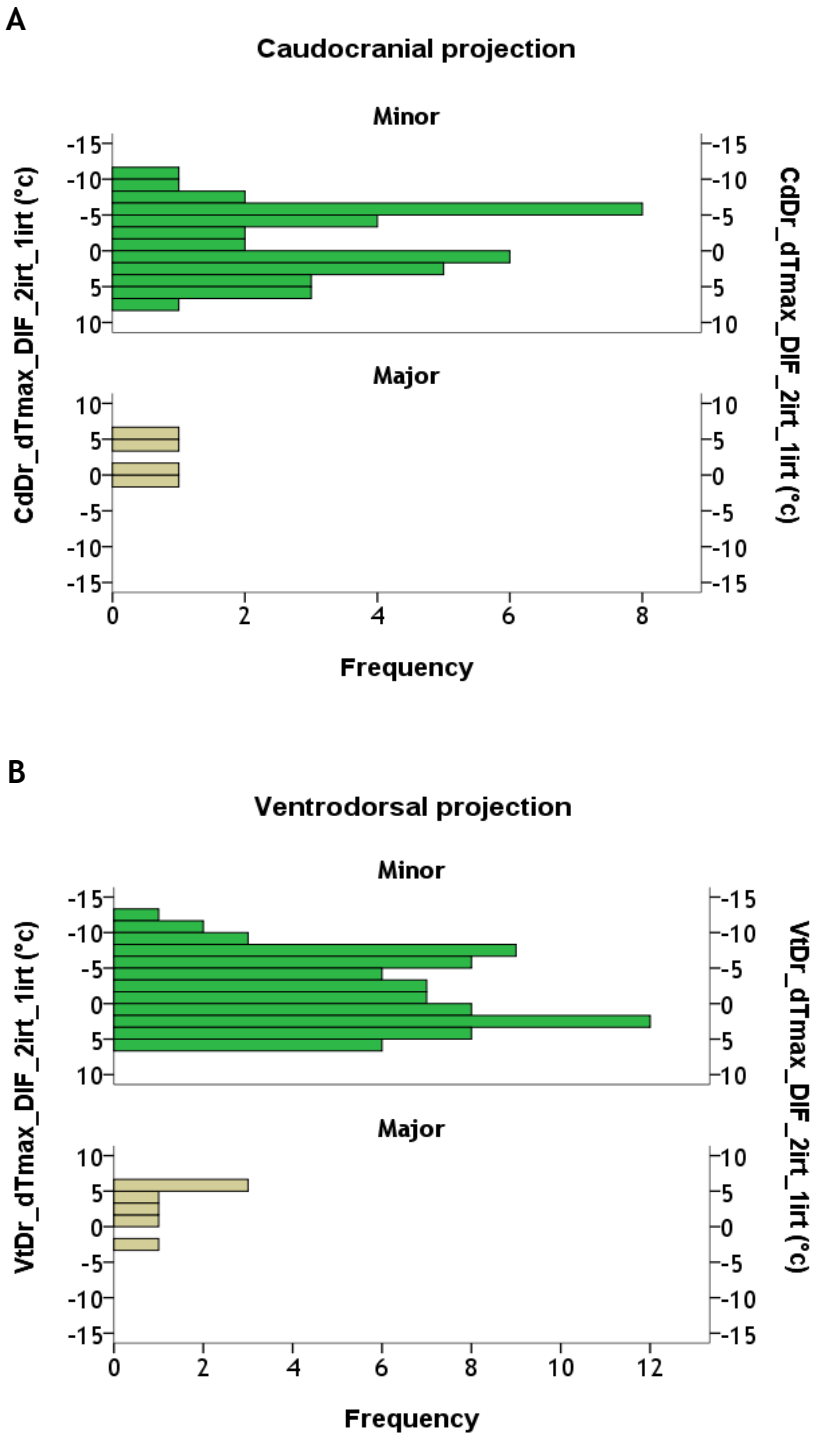

Figure 6-14 Temperature changes for $dT_{'max'}$ between first and second IRT pre-partum in quarters that were classified as infected with minor or major pathogens at subsequent calving.

6.2.3.2 IRT temperature by somatic cell count at first milking

Quarter and teat maximum temperature gradients ($dT_{'max'}$) and teat orifice temperature gradient ($dT_{'spot'}$) were also analysed to determine possible associations with SCC1 (Figures 6-15 and 6-16). Caudocranial projections did not show a significant association between quarter or teat temperature and SCC, but ventrodorsal projections did, both at 1st IRT ($p = 0.009$ and $p = 0.015$, for quarters and teats, respectively) and at 2nd IRT ($p = 0.003$ and $p = 0.002$ for quarters and teats, respectively). Details of median temperatures, temperature ranges and statistical comparisons can be found in Appendix 3.

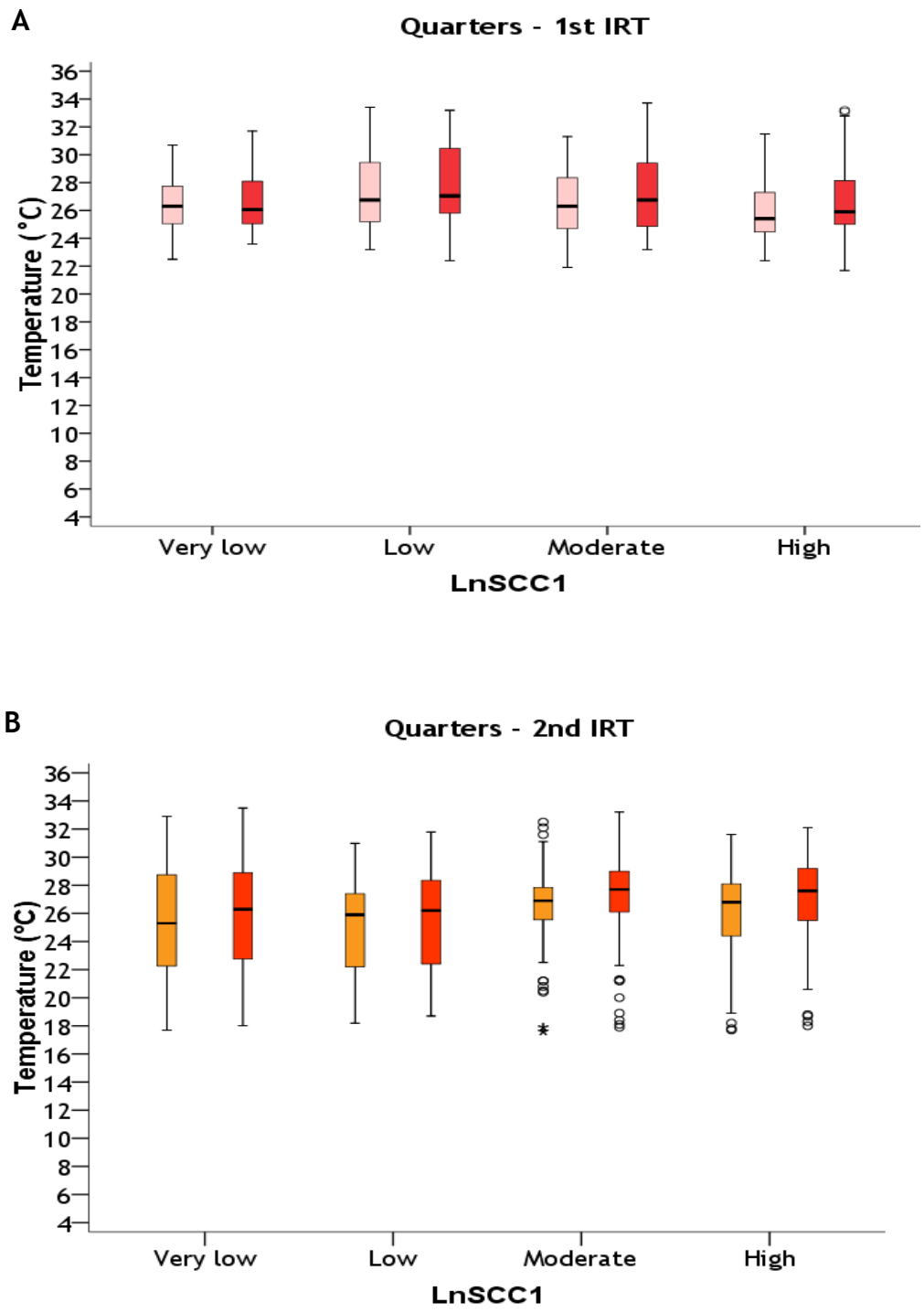

Figure 6-15 Boxplots comparing the distribution of quarter IRT temperature ante partum by natural logarithm of somatic cell count at calving (LnSCC1). A - first IRT: ▪ dT'max' at craniocaudal projection; ▪ dT'max' at ventrodorsal projection. B - second IRT: ▪ dT'max' at craniocaudal projection; ▪ dT'max' at ventrodorsal projection.

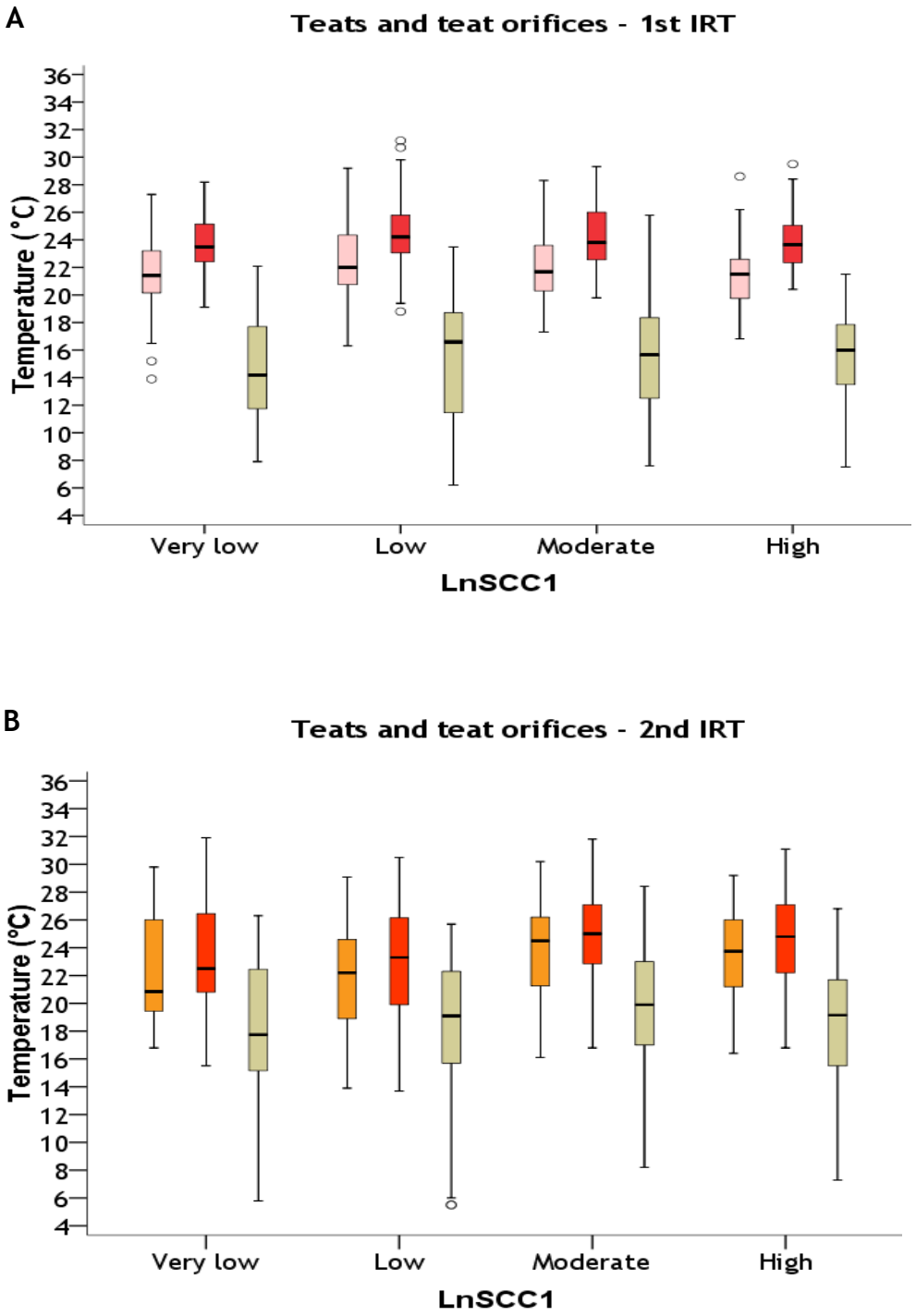

Figure 6-16 Boxplots comparing the distribution of teat and teat orifices IRT temperature ante partum by natural logarithm of somatic cell count at first milking (LnSCC1). A - first IRT: ▪ dT'max' at craniocaudal projection; ▪ dT'max' at ventrodorsal projection; ▪ dT'spot' (teat orifice). B - second IRT: ▪ dT'max' at craniocaudal projection; ▪ dT'max' at ventrodorsal projection; ▪ dT'spot' (teat orifice).

6.2.4 IRT as indicator of IMI at first day of lactation

An initial ROC analysis was conducted to assess the performance of single temperature parameters as a diagnostic test to discriminate between infected and non-infected quarters. The area under the ROC curves (AUROC) for each individual temperature parameter of each projection indicated that none of the parameters is a useful single indicator of IMI of dairy heifers at calving, as it can be seen by the proximity of all ROC curves to the reference line that indicates non-informative test and leading to AUROC curves close to 0.5 (Fig 6-17 and 6-18, Table 6-6 and 6-7 for temperature gradients and surface temperatures, respectively). Additionally, AUROC values for dT and Ts parameters were not statistically significant (Table 6-6 and 6-7 respectively), except $dT_{'max'}$ for quarter and teat skin surface at first IRT (2 months ante partum) ventrodorsal projection (Table 6-6).

Table 6-6 Test performance of individual gradient (dT) parameters measured by infrared thermography (IRT) at 2 months (1st IRT) or 2 weeks (2nd IRT) antepartum as indicators of IMI at calving.

Projection	Body surface temperature gradient	1st IRT			2nd IRT		
Quarter - 'areas'		AUROC	95% CI	*p-value*	AUROC	95% CI	*p-value*
Cd-Cr	$dT_{'min'}$	0.43	0.34-0.53	0.180	0.49	0.39-0.59	0.871
	$dT_{'avg'}$	0.46	0.36-0.56	0.418	0.43	0.33-0.53	0.150
	$dT_{'max'}$	0.43	0.33-0.52	0.120	0.44	0.34-0.54	0.241
Vt-Dr	$dT_{'min'}$	0.48	0.38-0.57	0.618	0.44	0.34-0.53	0.202
	$dT_{'avg'}$	0.44	0.34-0.54	0.228	0.46	0.35-0.56	0.374
	$dT_{'max'}$	0.40	0.30-0.49	0.037*	0.45	0.35-0.55	0.317
Teat - 'line'							
Cd-Cr	$dT_{'min'}$	0.44	0.34-0.53	0.207	0.45	0.34-0.56	0.296
	$dT_{'avg'}$	0.44	0.34-0.52	0.178	0.46	0.35-0.56	0.390
	$dT_{'max'}$	0.46	0.29-0.49	0.307	0.46	0.35-0.56	0.405
Vt-Dr	$dT_{'min'}$	0.46	0.36-0.55	0.403	0.44	0.33-0.54	0.182
	$dT_{'avg'}$	0.46	0.36-0.54	0.343	0.43	0.33-0.54	0.161
	$dT_{'max'}$	0.40	0.29-0.49	0.028*	0.44	0.33-0.54	0.181
Teat - 'spotmeter'							
Vt-Dr	$dT_{'spot'}$	0.47	0.37-0.56	0.449	0.45	0.34-0.55	0.290

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal. *Significance level at $p < 0.05$

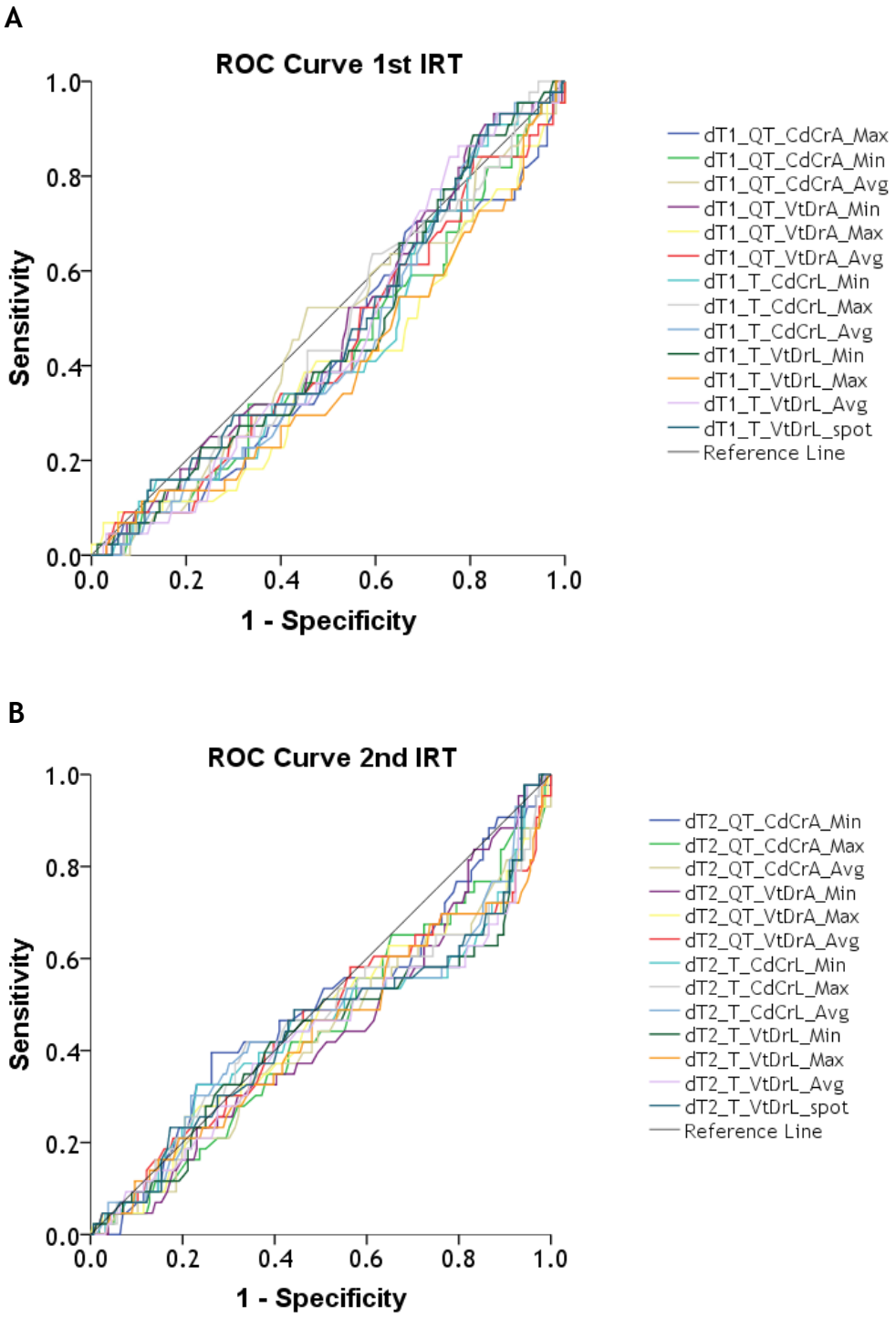

Figure 6-17 Receiver operating characteristic (ROC) curves of temperature gradients (dT) for detection of intramammary infection (IMI) in dairy heifers. A - 1st IRT; B - 2nd IRT. dT1 - temperature gradient at 1st IRT; dT2 - temperature gradient at 2nd IRT; QT - quarter; T - teat; CdCr - caudocranial projection; VtDr - ventrodorsal projection; A_Min - minimum area temperature; A_Max - maximum area temperature; A_Avg - average area temperature; L_Min - minimum line temperature; L_Max - maximum line temperature; L_Avg - average line temperature; L_spot - spot temperature of teat orifice.

Table 6-7 Test performance of individual surface (T_s) parameters as indicators of IMI at 1st and 2nd IRT.

Projection	Body surface temperature gradient	1st IRT			2nd IRT		
Quarter - 'areas'		AUROC	95% CI	*p-value*	AUROC	95% CI	*p-value*
Cd-Cr	$T_{s'min'}$	.51	0.42-0.60	0.897	0.56	0.46-0.65	0.264
	$T_{s'avg'}$	.57	0.48-0.66	0.143	0.53	0.43-0.63	0.577
	$T_{s'max'}$	.58	0.49-0.67	0.119	0.56	0.46-0.67	0.263
Vt-Dr	$T_{s'min'}$	.51	0.45-0.63	0.466	0.49	0.40-0.59	0.844
	$T_{s'avg'}$	.56	0.47-0.66	0.192	0.55	0.41-0.61	0.292
	$T_{s'max'}$	.56	0.47-0.65	0.240	0.53	0.41-0.60	0.569
Teat - 'line'							
Cd-Cr	$T_{s'min'}$	0.51	0.42-0.60	0.207	0.52	0.42-0.62	0.630
	$T_{s'avg'}$	0.52	0.48-0.66	0.178	0.56	0.47-0.66	0.205
	$T_{s'max'}$	0.54	0.49-0.67	0.307	0.57	0.47-0.66	0.184
Vt-Dr	$T_{s'min'}$	0.52	0.43-0.61	0.403	0.50	0.40-0.60	0.995
	$T_{s'avg'}$	0.53	0.44-0.62	0.343	0.51	0.41-0.61	0.818
	$T_{s'max'}$	0.53	0.44-0.62	0.028*	0.50	0.41-0.60	0.956
Teat - 'spotmeter'							
Vt-Dr	$T_{s'spot'}$	0.51	0.42-0.61	0.449	0.53	0.44-0.63	0.512

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal. *Significance level at $p < 0.05$

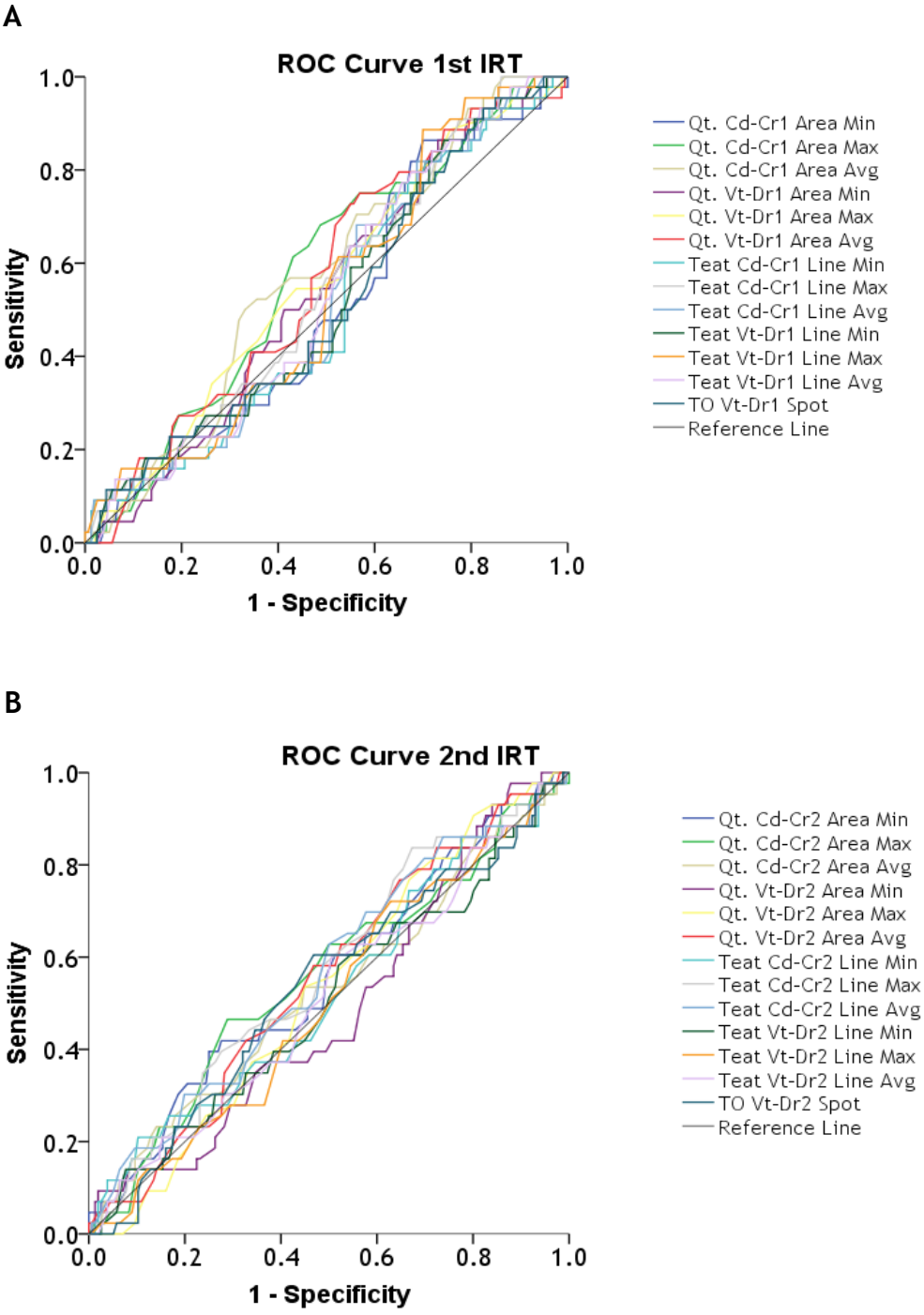

Figure 6-18 Receiver operating characteristic (ROC) curves of surface temperatures (Ts) for detection of intramammary infection (IMI) in dairy heifers. A - 1st IRT; B - 2nd IRT; QT - quarter; TO - teat orifice; CdCr - caudocranial projection; VtDr - ventrodorsal projection; Min - minimum area temperature; Max - maximum area temperature; Avg - average area temperature; spot - spot temperature of teat orifice.

A binomial logistic regression was performed to ascertain the association between $dT_{'max'}$ of each projection and IRT time and the likelihood that quarters have IMI. All continuous independent variables (1st and 2nd IRT $dT_{'max'}$ temperatures of craniocaudal and ventrodorsal projections) were found to be linearly related to the logit of the dependent variable. The logistic regression of each of the four models indicating that none of the predictors ($dT_{'max'}$ variables) were associated with IMI status at calving (Table 6-8).

Table 6-8 Omnibus tests of model coefficients for final models describing the association between infrared thermography (IRT) measurements at 2 months (1st IRT) and 2 weeks (2nd IRT) pre-partum and intramammary infection status at calving.

1st IRT	Cd-Cr projection	$\chi^2(2) = 3.17$, $p = 0.205$
	Vt-Dr projection	$\chi^2(2) = 2.13$, $p = 0.546$
2nd IRT	Cd-Cr projection	$\chi^2(2) = 2.37$, $p = 0.305$
	Vt-Dr projection	$\chi^2(2) = 3.01$, $p = 0.391$

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; significance level at $p < 0.05$

6.2.5 IRT as indicator of SCC at first day of lactation

An ordinal logistic regression was run to determine the association between $dT_{'max'}$ of each projection and IRT time and the somatic cell count categories at first milking. The ordinal regression of each of the final four models was not statistically significantly (Table 6-9), indicating that the final models were not reliably predicting SCC based on lnSCC at first milking.

Table 6-9 Likelihood-ratio test for final models describing the association between infrared thermography (IRT) measurements at 2 months (1st IRT) and 2 weeks (2nd IRT) pre-partum and SCC at calving.

1st IRT	Cd-Cr projection	$\chi^2(2) = 1.58$, $p = 0.455$
	Vt-Dr projection	$\chi^2(3) = 1.80$, $p = 0.616$
2nd IRT	Cd-Cr projection	$\chi^2(2) = 2.98$, $p = 0.226$
	Vt-Dr projection	$\chi^2(3) = 6.23$, $p = 0.101$

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; significance level at $p < 0.05$

7 Discussion

The aim of this study was to assess the usefulness of IRT as tool to assess udder surface temperature in dairy heifers under field conditions and to evaluate its potential to predict udder health as defined by IMI and elevated SCC at parturition. To the author's knowledge no other study has used IRT as a non-invasive tool to assess udder health in nulliparous dairy heifers on-farm conditions.

7.1 Use of IRT on heifers under field conditions

Although IRT technology has been applied to diagnose inflammatory conditions in veterinary medicine, including experimentally induced mastitis, little information is available about IRT use under field conditions to detect IMI without CM. Field studies using IRT are difficult due to diverse factors that can affect temperature readings by thermal cameras, such as air ambient temperature, wind drafts, sunlight exposition and humidity (Stewart *et al.*, 2005; Rekant *et al.*, 2016). These factors were taken into consideration in the IRT collection technique in this study and their impact was minimized by the protocol of IRT image collection, e.g. avoiding increase in udder surface temperatures due to the direct exposition to sunlight (especially important in dark pigmented skin udders), intensive exercise or brushing of the udder skin, or decrease in udder surface temperatures due to wet udder skin or cold wind drafts. Thus, in this study the protocol included the collection of IRT images at constant distance between udder surfaces and lens of the thermal camera and lower than one meter, because gases in the air (e.g. CO_2) absorb more infrared radiation from the udder surface as the distance increases (Okada *et al.*, 2013); the angle of the camera lens with the udder surface, which was demonstrated not having significant differences when set within 45 degrees to the object (Okada *et al.*, 2013); image collection was performed after 20 - 30 min of animals handling and previous to any other animal data collection; images were collected in a routine handling area with no wind drafts or reflective surfaces or direct sunlight. In addition, all IRT image collection was performed at the same time of the day to minimise the effect of circadian thermal variations, which can present a variation in temperature of 0.34°C (Lefcourt *et al.*, 1999; Salles *et al.*, 2016). To make the IRT as routine tool feasible, similar conditions would need to

be established on commercial dairy farms. This could potentially be achieved by conducting IRT measurements on groups of animals at the entrance of a milking parlour (e.g. setting a protocol for a specific milking time) or an in-house cattle race after resting at the collecting yard and avoiding of rushing cattle into them. Whilst data collection was possible without causing undue stress to heifers, considerable operator training was required for set-up for data collection and also for data analysis. This decreases the likelihood that IRT will become a tool for routine use on dairy farms.

Ambient air temperatures records from previous years were checked on the MetOffice website prior to on-farm data collection to ensure the instrument's calibration covered the expected temperatures in the months of IRT collection. In addition, ambient air temperature was measured at the time and point of IRT image collection, allowing for detection of an impact of ambient air temperatures on surface temperatures measure by IRT, with 10% to 52% of the variability of udder surface temperatures explained by its relationship to the ambient temperature (Fig 6-2 and Table 6-4). The impact of ambient air temperature and humidity on heat exchanges in hot or cold environment is well recognized (Hahn *et al.*, 2009) and several strategies have been used to minimise its influence in IRT data. In line with standard methodology for IRT, the gradient temperature was used in this study, as obtained from the comparison between surface temperature and ambient air temperature (McCafferty *et al.*, 2013; Mellish *et al.*, 2015). The need for such corrections does add to the need for user training and limits the usefulness of IRT as a point-of-care test on dairy farms.

The hair coat of the udder was not trimmed in our study to reflect the situation on most UK dairy farms, where hair removal of the udder may be used in lactating cattle but rarely in nulliparous cattle. This is an important aspect in our study, because the quantity of coat can interfere with the readings of the thermal camera. Udder hair was not brushed or washed to avoid the effect of the procedure on the thermal images, it was not feasible as part of the farm routine and because the degree of dirt on the skin was very low. However, it cannot be excluded that it could have some impact on the temperature data

collected, with a some retention of air interfering with the exchange of heat between udder skin and ambient environment (Lynch *et al.*, 2012). For example in Australian fur seals it was found a mean difference of 6.6°C between alopecic and nonalopecic areas (Lynch *et al.*, 2012). The type of hair coat of these animals is obviously different from cattle, but it would be interesting to further investigate the degree of difference between clipped and unclipped udders and different densities of coat to allow the determination of a correction factor to be applied on the software of IRT analysis, as for example the introduction of ambient temperature and humidity. However, the need for clipping would detract from the practicality of routine IRT use on farms.

Core temperatures as assessed by rectal temperature measurements presented means within the thermoneutral zone for adult cattle (38.6°C - 39.5°C) (Radostits *et al.*, 2000). At first IRT, individual rectal temperatures were slightly higher than at second IRT (Fig 6-1). Values outside the thermoneutral range without overt signs of disease were observed at first and second IRT. Rectal temperature is commonly used during clinical examination to assess internal body temperature; it is considered fast, affordable and minimally invasive, but it may be influenced by stress responses (McCafferty *et al.*, 2015). Stress can induce hyperthermia as well as an increase of production of metabolic heat associated to the escape response. In mice, rectal temperature may increase by 0.5°C to 1.5°C in 10 to 15 minutes due to the increase of corticotrophin release hormone and marked autonomic changes under stress situations. In addition, the escape response may include elevation of arterial pressure and intense heart rate (Bouwknecht *et al.*, 2007; Busnardo *et al.*, 2010). If similar processes occur in cattle, this may explain why some individual rectal temperatures were higher than the normal range for adult cattle. Heifers at 2 months pre-partum have less regular contact with people than those at two weeks pre-partum, when they are located at the close-up pen, which could explain the higher temperatures. In addition, there may be differences in physiology, and there are reports of slightly higher body temperature in pregnant and rapidly growing animals (Wrenn *et al.*, 1958). Finally, any normal data distribution will include unusually low or high values at the tail end of the distribution. Those extreme values may fall below standard cut-off values for normal body temperature, even if they are

part of the normal range. In my study, rectal temperatures were measured after IRT image collection to minimise the chances of udder surface temperature be affected by stress caused by touch, holding the tail and insertion of thermometer.

The last trimester of gestation is a period of increased risk of IMI (Sordillo and Streicher, 2002; Nyman *et al*., 2009), which my manifest at calving or in early lactation as CM or elevated SCC. The two times of IRT image collection in the current study were selected within this period, representing the pre-colostrogenesis and colostrogenesis phases. Synchronization of data collection with routine animal handling reduced disruption in the farm work and limited the impact on animal welfare. These time points were furthermore selected to avoid the higher risk period for occurrence of udder oedema, which increases closer to the calving day, and to minimize interference with heifers that are in preparation for calving, which could lead to premature or delayed calving, as might be the case if data were collected one week and one day before calving respectively. Other practical considerations, which are important for evaluation of a test under the conditions where it might be used in practice, include the fact that the exact calving date is difficult to predict, and that it is easier to screen animals in groups than individually.

In most studies in cattle, IRT images were taken caudocranially for the back quarters and laterally for the front quarters (Berry *et al*., 2003; Colak *et al*., 2008; Hovinen *et al*., 2008; Polat *et al*., 2010; Pezeshki *et al*., 2011; Metzner *et al*., 2014; Sathiyabarathi *et al*., 2016b). However, those studies involved primiparous and multiparous cows that have bigger udders, which are easier to assess laterally than in nulliparous dairy cattle. Because of the impossibility of taking lateral projections to assess the front quarters, due to the small size of the immature mammary glands at first IRT and the use of head yokes with animals side-by-side at second IRT, ventrodorsal projections were the solution explored to assess the surface temperatures of those quarters. To the author's knowledge, this is the first study using this projection to assess the udder temperature of dairy cattle. The only study found with similar approach to the udder was realised in dairy sheep in Brazil (Martins *et al*., 2013).

The polygons geometric tool was the best tool to detect significant changes in the udder temperature in cattle with induced *E. coli* mastitis (Metzner *et al.*; 2014). Because of this and considering that it covered a larger area of udder skin, polygons rather than lines were used for the analysis of quarter IRT images in this study. Because of the curved surface shape of the teats, data from teats was gathered with lines tool, after visualisation of the teat with the polygon tool. Ideally, this could be simplified by the use of automated evaluation with IRT software with algorithms that recognize the udder contours and exclude the border areas and intermammary grove (Metzner *et al.*, 2014). Careful consideration of these aspects during analysis of IRT images helps reducing errors in temperature measurements due to the curvature of the udder, proximity to the medial aspect of the rear legs and shape of the intermammary groove, but when realized manually is laborious (Metzner *et al.*, 2014). Even with a careful manual setting of the geometrical analysis tools, by a single operator, it is possible that some imprecision might be introduced. This is another feature of IRT that limits its usefulness for routine on-farm application.

The results of the comparison between caudocranial and ventrodorsal projections showed high potential of the latter to assess all quarters' maximum temperatures at both times of IRT collection (Fig 6-3 and Fig 6-4). This finding was considered a relevant result in terms of applicability of the tool on-farm, in cattle races or milking parlours. An advantage of the ventrodorsal approach of the udder is that it allows acquisition of a representative IRT image of all quarters without causing disturbance to the animal. The very strong correlation with projections of the udder skin surface agrees with the findings of Okada *et al.* (2013), who reported no effect of the use of 45 degrees angle between camera lens and target surface on IRT image quality. At first IRT, teats' projections presented higher correlation for $dT_{'min'}$ than for $dT_{'max'}$ whereas at second IRT the quarters presented similar difference with higher correlation for $dT_{'max'}$ than for $dT_{'min'}$. The author hypothesises that the lower correlation of the teats' $dT_{'max'}$ might be the result of its smaller length at first IRT (2 months antepartum), which in the ventrodorsal projection might suffer interference of a greater proximity to the quarters in the IRT image (higher $T_{s'max'}$ at ventrodorsal projection and $T_{s'max'}$ interquartile range at caudocranial projection Table 6-1),

similar to the proximity effect of the medial aspect of the rear legs to the lateral aspect of the udder (Metzner *et a*l., 2014). The lower correlation of quarters' $dT_{,min,}$ at second IRT (2 weeks antepartum) might be the consequence of differences in the degree of dirt on the skin surface of the quarters as result of a more developed and lower udder than at first IRT, with a larger surface area exposed to the ambient air temperature and contamination (higher $T_{s,min,}$ interquartile range at caudocranial projection Table 6-2), or even some degree of udder oedema interfering differently with the IRT image of each projection.

Core temperature (rectal temperature) and maximum udder surface temperatures measured by IRT were only very weakly to moderately correlated (Table 6-3), in contrast to results reported in other studies for non-infected and experimentally infected quarters (Metzner *et al*., 2014). At first IRT, $dT_{,max,}$ from quarters and teats in both projections were negatively correlated with rectal temperature. Interestingly, at second IRT, $dT_{,max,}$ were positively correlated with rectal temperature. The results at first IRT might reflect the development status of the udder. Conversely, increased blood flow at the time of colostrogenesis and close to parturition (Thompson, 1980) could explain the results observed at second IRT.

The collection of IRT images is easy, non-stressful and fast, however the application of this non-contact technique on-farm requires development of the analysis software to be automated, with the possibility of a quick reading of corrected surface temperatures given for example by complex algorithms as used for the investigation of breast tumours in women (Tan *et al*., 2009), to be attractive for use on farm environment by farmers. Although the potential value of IRT as indicator of udder health appeared low, as discussed below, ongoing developments in IRT technology may improve diagnostic accuracy, as exemplified by the recent introduction of vital-sign-based IRT for respiratory disease (Sun *et al*., 2017).

7.2 Use of IRT as indictor of udder health

The unit of analysis in this study was the quarter, which was considered an independent unit with equally probability of getting infected (Sol *et al.*, 2000; Berry and Meaney, 2006; Nickerson and Akers, 2011). This option was taken in recognition of the separate physiological status of quarters within an animal and to maximize the number of units observed, as considered in previous mastitis studies (Zadoks *et al.*, 2001). Other reason for the use of quarter level samples instead of heifer level was the possibility of under-detection of quarters with high SCC due to dilution by colostrum from uninfected quarters with low SCC in composite samples (Barkema *et al.*, 1999), the fact that the prognosis for cure from IMI depends on the number of infected quarters (Barkema *et al.*, 2006) and the fact that most mastitis treatments consist of injectors that are applied at quarter level. In the statistical analysis, correction for clustering of quarters within heifers was not used. This does not affect parameter estimates, but it violates the assumption of independence of observations that underpins many statistical analysis, and could results in type I errors, i.e. declaring significance when none exists. Clustering limits variability and reduces the noise to signal ratio and hence reduces *p*-values, i.e. increases apparent significance. Given that hardly any significant associations between IRT data and udder health indicators were identified in this study, type I errors are not of concern here. Comparison of infected quarter prevalence at calving with other studies needs to be done cautiously because definition of gland status based on bacteriological culture vary considerably between studies (Sargeant *et al.*, 2001, Dohoo *et al.*, 2011). In addition, apparent and true prevalence may be affected by sample methodology and risk factors that are specific to a production system or individual farm (Pankey *et al.*, 1991; Fox *et al.*, 1995; Nickerson *et al.*, 1995). In line with results from expert group consultation, we defined IMI based on the presence of 10 or more CFU per plate of a bacterial morphotype, the equivalent to ≥1000 CFU per millilitre, in quarter level samples (Andersen *et al.*, 2010).

The colostrum samples were frozen at -20°C immediately after sampling to decrease the probability of bacterial overgrowth by contaminants (Barkema *et al.*, 1997). Storage of milk samples at -20°C for up to 12 weeks does not adversely affect the retrieval of major pathogens (Murdough *et al.*, 1996; Petzer

et al., 2012). Therefore, the storage of colostrum samples up to 3 weeks in this study is unlikely to have contributed to the low prevalence of major pathogens in this study. The proportion of quarters infected (20%) was similar to the result obtain by Andrew (2001), who reported 17% infected quarters based on testing of heifer colostrum samples. The majority of infected quarters tested positive for minor pathogens, but major pathogens were also isolated (Fig 6-6B). The prevalence of minor pathogens, particularly CNS in samples from dairy heifers at calving and in the early postpartum period, has been reported in a large number of studies (Myllys and Rautala, 1995; Pankey *et al.*, 1991, 1996; Aarestrup and Jensen, 1997; Borm *et al.*, 2006; Sampimon *et al.*, 2009a; Piepers *et al.*, 2010; Piepers *et al.*, 2011; De Vliegher *et al.*, 2012). The low percentage of colostrum samples that was contaminated (3%) demonstrated that well-trained farm staff can collect high quality samples that are as good as those collected by specialized technicians (Scherpenzeel *et al.*, 2014). This is important for the transition from laboratory-based to cow-side diagnostic tests for herd health screening and monitoring.

In lactating cows, back quarters often have higher SCC than front quarters, which is attributed to faecal contamination and opportunistic IMI. Significant differences can also be found between left and right quarters associated with lying behaviour and location of the rumen (Barkema *et al.*, 1997). In this study, there was no evidence of a difference between front and rear or left and right quarters. This could be due to good management and comfort of bedding material at the farm, as reflected in the high cleanliness of the udders during sample collection (IRT images and colostrum samples) and due to the smaller development of the mammary glands which pose a smaller risk for contamination due to contact of the udder with dirty bedding than in adult cows. It is also unlikely that this study would have had the statistical power necessary to detect such differences, which are visible in large data sets from DHI records. Similar to a study by Barkema *et al.* (1999), our data showed that quarter SCC at calving can be low (Table 6-5 and Fig 6-7). SCC in infected quarters was significantly higher median than in culture-negative quarters or quarters with low CFU but there was no significant difference between culture-negative quarters and quarters with low CFU. These results show that low-level culture-positivity at

calving (100 to 1,000 CFU/ml) is not necessarily associated with inflammation of the mammary gland and that infection prevalence in heifers at calving may be overestimated when inflammatory markers are not considered. Likewise, SCC may be a poor indicator of inflammation because it is difficult to define accurate threshold due to physiological factors that can increase SCC during the first days postpartum (Hariharan *et al.*, 2004; Bludau *et al.*, 2014). High SCC at calving has been attributed to an excessive desquamation of epithelial cells in a small volume (Schalm *et al.*, 1971) and a higher percentage of neutrophils than in milk or dry cows mammary secretion (Sharma *et al.*, 2011). Other possibilities for high SCC in milk that is culture-negative on blood agar is the presence of *Mycoplasma* or variable bacterial shedding (Hariharan *et al.*, 2004). There were no clinical or historical reasons to suspect a role of *Mycoplasma* in heifer – or udder health on the study farm. Median SCC was significantly higher in quarters infected with minor pathogens (Fig 6-9), where CNS were predominantly isolated, compared to culture negative quarters. CNS are described by some researchers as an emerging cause of subclinical mastitis (Pyörälä and Taponen, 2009). IMI quarters presented a trend to increase SCC with the increasing number of CFU per plate, however the differences were not statistically significant, probably reflecting lack of power (type II error) rather than the absence of biological difference (Fig 6-8).

The analysis of $dT_{,max,}$ temperatures at 1st IRT ventrodorsal projection showed apparent differences between culture status, with infected quarters presenting lower median $dT_{,max,}$ than low CFU or culture-negative quarters (Fig 6-10A), albeit the lack of statistically significance supporting this observation, it can indicate a temperature pattern. Similarly, quarters infected with major pathogens (Fig 6-12A) and quarters with higher SCC (Fig 6-15A) presented lower $dT_{,max,}$ temperatures compared with other groups in analysis, however these results were statistically significant adding indications for a lower temperature pattern in the quarters with the likely worst outcome (Appendix 3). IMI may cause lower temperature, whereby decreased functionality and poor blood circulation to the quarter are caused by pathogens (McGavin and Zachary, 2007; Zhao and Lacasse, 2008), or, conversely lower temperature of the udder may reflect lower blood circulation (Thompson, 1980) and consequently poorer

immune surveillance, making the quarter more susceptible to IMI and particularly to major pathogens. Although an association between disease and temperature increase may seem counterintuitive, similar findings were reported in a study conducted in adult dairy cows (Bortolami *et al*. 2015). This is in contrast to other studies on IRT and mastitis, which showed an increase in udder skin surface temperature along with higher SCC results and high sensitivity and specificity of IRT to detect subclinical mastitis in cattle (Polat *et al*.; 2010). Likewise, Martins *et al*. (2013) were able to correctly classify sheep into different mastitis groups based on udder temperature data, as well as clearly differentiate the group of subclinical mastitis, using a ventrodorsal approach of the udder.

The lack of diagnostic value of IRT to detect IMI and subclinical mastitis was supported by AUROC analysis (Fig 6-17 and Fig 6-18, Table 6-6 and Table 6-7, respectively) and the logistic regression analysis. These results agree with the initial perception given by the considerable heterogeneity in the dT'_{min} and dT'_{max} values in Fig 6-3 and Fig 6-4, which suggested a limited sensitivity and specificity of any particular threshold value as indicator of udder health (intramammary infection and elevated SCC). However, the number of major pathogens isolated was low may have affected the ability to observe an effect. A study using IRT technology in a farm with a greater number of heifers infected with major pathogens would benefit the understanding of udder surface skin temperature in quarters infected with these pathogens. The potential of this technology to predict CM at calving was not explored because CM was not observed at calving. This may be the result of the herd management measures put in place after high incidence of mastitis in early lactating heifers in the previous years. Further studies in farms with a high incidence rate of CM at calving are recommended to understand if the pre-partum IRT ventrodorsal projection has potential to predict CM in nulliparous dairy heifers.

8 Conclusion

IRT is a non-invasive method, which allows for fast and contactless collection of udder surface temperature data, but current software for IRT image analysis restricts the use of this technology to research or individual cases.

Ventrodorsal projection of the udder allows for data collection from all quarters and may be advantageous in a farm setting, where it could be incorporated on the floor of cattle crushes, races or milk parlours for early detection of clinical mastitis, which might be useful if further development software allows automatic temperature readings of a specific area of a cow and provided that biologically meaningful cut-off alarm points can be identified to select cows for further examination. The current study did not provide evidence for the value of IRT as a screening tool for early detection of intramammary infection with minor pathogens in nulliparous dairy heifers. However, it showed an unusual thermal pattern in quarters that presented high SCC, major pathogens and higher CFU, which might deserve further research to better understand udder skin temperature in heifers with abnormal udder health. Considering the value of heifers to dairy farms, increasing specialization and automation in dairy farming and heifer rearing, and societal pressure to reduce the use of antimicrobials for treatment or prevention of IMI, it may be worth revisiting the use of IRT periodically as more sophisticated equipment and software become available.

Appendices

Appendix 1 – Milk haptoglobin study in the subset of heifers

Research in Veterinary Science xxx (2017) xxx-xxx

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com

Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving

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ARTICLE INFO

Keywords:
Heifer
Biomarker
Intramammary infection
Haptoglobin
Postpartum
Somatic cell count

ABSTRACT

Mastitis, inflammation of the mammary gland, is often caused by intramammary infection with bacterial organisms. It impacts on dairy cattle welfare, production, udder health and longevity in the herd. Current detection methods for mammary inflammation and infection all have limitations, particularly for on-farm diagnosis of non-clinical mastitis after calving. Acute phase proteins have been suggested as alternative early indicators of the disease and can potentially be used as cow-side test with results in real time. In this study, milk haptoglobin concentrations were investigated over the first week postpartum to explore haptoglobin's potential as indicator of udder health in dairy heifers. Haptoglobin concentration was highest on day 3 of lactation, and was positively correlated with somatic cell count, a commonly used marker of inflammation (r_s = 0.68). Haptoglobin level was also associated with bacteriological culture results, a key indicator of infection status, whereby median haptoglobin concentration on days 3 and 5 was higher in quarters that were infected at calving than quarters that were non infected at calving. Sensitivity and specificity of haptoglobin concentration as indicator of infection were low, both for lenient and strict culture-based definitions of intramammary infection (57 or 60% and 61 or 63%, respectively). Although haptoglobin was a poor biomarker for intramammary infection with coagulase negative staphylococci in heifers during the first week after calving, it may have value as an indicator of major pathogen infections, particularly in large scale dairy herds where pre-partum heifers are managed off-site.

1. Introduction

Mastitis, inflammation of the mammary gland, is one of the most common diseases affecting dairy cattle. It is well recognized as a serious production problem and a costly disease (Halasa et al., 2007) due to the direct effect on milk yield, composition and processing properties (Auldist et al., 1996), and the impact on animal welfare and its association with a higher culling risk (De Vliegher et al., 2012).

In recent years, the intensification and technological progress in dairy farming has resulted in major changes to herd management and milking methods. Large herds often rely on off-site contracted heifer-rearing units to raise their replacement animals. Information regarding growth rate and reproductive performance of heifers in such units is frequently shared with the main herds, but udder health status and hygiene history are largely unknown. The transfer of these heifers to the main herd generally occurs during the last months of gestation. This transfer coincides with major physiological changes related to the approach of parturition and beginning of the lactation, which is recognized as a period of increased susceptibility to udder health problems (De Vliegher et al., 2012; Sordillo, 2005). Damage to the mammary tissue during this period may result in long term adverse effects on udder health and milk yield, which affect dairy farming profitability and sustainability (De Vliegher et al., 2012).

For decades, udder health has primarily been evaluated using detection of mammary inflammation, e.g. through somatic cell counting, or intramammary infection, e.g. using culture-based methods. Modern advances in proteomics have led the way to the discovery of biomarkers

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http://dx.doi.org/10.1016/j.rvsc.2017.05.024
Received 24 February 2017; Received in revised form 4 May 2017; Accepted 22 May 2017
Available online xxx

that have the potential to detect udder health problems earlier, in-line , faster, less expensively and with higher sensitivity (Viguier et al., 2009). Acute Phase Proteins (APPs) are a group of biomarkers with potential as indicators for udder health (Py r l et al., 2011). These proteins are mainly produced in the liver and concentrate in blood as a result of the acute phase response, a cascade of events that occurs after an inflammatory process is triggered, for example by bacterial invasion of the mammary gland (Petersen et al., 2004). APP are recognized as integral players in the mammary gland's innate immunity, responding non-specifically to pathogenic invasion (Cray et al., 2009). Their concentration in milk from healthy animals is negligible but it is significantly elevated in milk from cows with clinical or subclinical mastitis, increasing the interest in their ability to differentiate healthy from infected quarters (Eckersall et al., 2001, 2006; Gr nlund et al., 2005). The fact that there is local expression of Hpt mRNA in mammary gland tissue, rather than influx of systemically produced Hpt, adds to the potential specificity of Hpt as marker of udder health (Eckersall et al., 2001; Hiss et al., 2004).

Reliable, sensitive and early detection of udder health problems is a crucial component of herd health and productivity management. Increased sophistication of milking systems, technical developments, and specialized, often highly trained staff provide new opportunities for on-farm, real-time udder health diagnostics (Viguier et al., 2009). The concentration of Hpt in milk is easier to measure than the concentration of other APP, and this measurement is possible using colostrum (Thomas et al., 2016b). For SCC, the level at calving and during the first days of lactation is related to heifers' IMI status at calving (Barkema et al., 1999). The wide range of Hpt values that can be observed at or shortly after calving, at least in adult cattle, (Thomas et al., 2016b) may also be indicative of udder health status. Therefore, the aim of this study was to investigate milk Hpt concentrations over the first week postpartum in dairy heifers, and the potential use of Hpt concentration as indicator of udder health.

2. Material and methods

2.1. Sample collection

The study was conducted on a commercial dairy farm with approximately 700 lactating animals on-site, and heifer rearing facilities off-site. Heifers were moved to the lactating cow facility approximately 6 weeks before expected parturition and housed in a heifer group. Shortly before calving, they were moved to a close-up and calving pen, which was also for heifers only. After calving, heifers were moved into the group of 1st lactation animals. All lactating animals were milked three times a day in a 52-point rotary parlour. Twenty-four healthy Holstein-Friesian heifers were followed during the first five days postpartum. Quarter level colostrum and milk samples were collected on the day of calving (day 1), and three (day 3) and five (day 5) days in milk. Samples were obtained from 96 quarters of 24 heifers over four weeks. Sample collection was conducted after teat disinfection with gauze swabs soaked in 70% ethanol and after the first streams of colostrum or milk had been discarded. On the day of calving, the farmer collected two samples from each quarter at first milking, i.e. during the colostrum phase. The first sample was collected into sterile pre-labelled 15 mL Falcon tubes (VWR international Ltd., UK) for bacteriological culture and stored on-farm at -20 C. The second sample was collected into pre-labelled 50 mL Falcon tubes (VWR international Ltd., UK) and split after collection. Approximately 20 mL were transferred into pre-labelled containers supplied by the National Milk Laboratories (NMR Co., Hillington Park, Glasgow) containing milk preservative (2-bromo-2-nitropropane-1,3-diol) and kept refrigerated at 4 C for up to 4 days, for somatic cell count measurement. The remainder of each sample was retained in the original 50 mL tube and stored at -20 C for haptoglobin analysis. On days 3 and 5, milk samples were collected for SCC and Hpt measurement only at the second milking of the day, starting at 11.30 am. The study was approved by the School Research Ethics Committee of the University of Glasgow, School of Veterinary Medicine (Ref31a/14).

2.2. Milk haptoglobin measurements

Colostrum and milk haptoglobin concentration was determined by sandwich ELISA procedure, using purified rabbit anti-bovine Hpt IgG (Life Diagnostics Inc., West Chester, Pennsylvania, USA) that was conjugated to alkaline phosphatase (Innova Biosciences, Cambridgeshire UK) following the manufacturer's instructions, with the assay procedure described previously (Thomas et al., 2015). The lower detection limit of the assay was 0.4 g/mL.

2.3. Somatic cell count measurements

Colostrum and milk samples were analysed at the National Milk Laboratories. SCC was determined by flow cytometry somatic cell counter technology (Fossomatic, Foss, Hiller d, Denmark) within 4 days of sample collection.

2.4. Bacteriological culture

All colostrum and milk samples were cultured within three weeks of sample collection, following the standards of the National Mastitis Council (Harmon et al., 1990). Samples were thawed under refrigeration for approximately 15 h, warmed to room temperature and mixed thoroughly. Then, 0.01 mL of each quarter sample was inoculated onto Sheep Blood Agar 5% plates (E&O Laboratories Limited, Bonnybridge, Scotland), using calibrated sterile plastic loops (Thermo Scientific Sterilin, UK). All plates were incubated aerobically up to 48 h at 37 C, and the number of colony-forming units (CFU) was counted for each bacterial morphotype. Quarters were considered culture-negative if no growth was observed. Samples containing three or more bacterial species were considered contaminated, and were not taken into account to define quarters as infected or non-infected. The remaining culture-positive samples were used to classify quarters as infected using a lenient and strict definition, respectively. For the lenient definition, any quarter with positive culture result in a non-contaminated sample was considered infected. This definition has high sensitivity but suboptimal specificity, i.e. 85.8% and 75.1% respectively for any organism (Definition A; Dohoo et al., 2011). For the strict definition, only quarters with at least 1000 CFU/mL of the same bacterial morphotype were considered infected. This definition has high specificity but suboptimal sensitivity, i.e. 100% and 35.2% respectively for any organism (Definition C; Dohoo et al., 2011). Definition A is recommended by Dohoo et al. (2011), whereas definition C is preferred by many mastitis experts (Andersen et al., 2010).

Gram stains, oxidase tests (Oxoid Ltd.), and catalase tests were carried out on all cultured bacterial isolates. Further identification was carried out based on the results of these tests. The tube coagulase test with rabbit plasma was used to differentiate coagulase positive and coagulase negative staphylococci, with the former presumed to be *Staphylococcus aureus*. Streptococci were differentiated from enterococci based on the absence of growth on MacConkey agar. Identification of streptococci to Lancefield group level was carried out using the Streptococcal Grouping Kit (Oxoid Ltd.). Streptococci that failed to react with antisera to groups A, B, C, or D were tested for aesculin hydrolysis and, if positive, assumed to be *S. uberis*. Quarters were classified as infected with minor (e.g. coagulase negative staphylococci and *Corynebacterium* spp.) or major (e.g. *S. aureus*, *S. uberis*) pathogens based on culture results (Anderson et al., 2010).

2.5. Data analysis

Analysis was performed at quarter level using Statistical Package for Social Sciences (SPSS) software version 22.0 (IBM SPSS, Portsmouth, UK). Variables were analysed using descriptive statistics, and normality was assessed using histograms, normal probability (Q-Q) plots and Shapiro-Wilk tests. Because of the non-normal distribution of the data, differences in median haptoglobin (Hpt) concentration and SCC between postpartum days were assessed by non-parametric tests (Friedman test for overall difference, with post-hoc Dunn-Bonferroni test to determine which groups differ). SCC and Hpt data was natural logarithm converted for data analysis and graphic presentation effects, however distribution was still non-normal. For each sampling day (1, 3, 5) the relation between milk Hpt concentration and SCC was examined visually and statistically using scatterplots and Spearman's rank correlation test, respectively. Hpt concentrations were compared between culture negative samples and those from infected quarters using boxplots using either lenient or strict definitions of infection. For each sampling day, differences between groups were assessed by Kruskal-Wallis H test and pairwise comparisons using Dunn's procedure with Bonferroni adjustment. Results were considered statistically significant at $P < 0.05$. Receiver operating characteristics (ROC) were analysed to assess test performance (area under the curve, AUC), cut-off values and respective sensitivity (*Se*) and specificity (*Sp*) for the diagnosis of infection status, using bacteriological culture as reference method (Dohoo et al., 2011). The selection of the cut-off values was made following the procedure described in Froud and Abel (2014) using Euclidean geometry to find the closest point of the ROC curve to the top-left corner of the ROC space, assuming that sensitivity and specificity should be valued equally.

3. Results and discussion

On large dairy farms, the udder health history and status of heifers that enter the lactating herd is often unknown, particularly when heifers are raised in rearing units that are separate from the main farm. Acute phase proteins may have value as biomarkers of udder health and the method for quantification of Hpt lends itself to pen-side test formats (Thomas et al., 2016a). Therefore, we investigated the value of Hpt concentrations in milk from dairy heifers in the first week post-partum as indicator of their udder health status.

Haptoglobin was present in moderate concentrations in colostrum samples (Table 1), in agreement with results reported by others (Thomas et al., 2016b). Thomas' (2016b) study did not provide information on the relationship between Hpt and infection status in the first week post calving, but our results show variable Hpt levels in samples from culture negative as well as infected quarters (Table 2). The presence of Hpt in colostrum from healthy mammary glands may be due to the antibacterial properties of APP and their role in passive immunity and protection of the newborn calf (Eckersall et al., 2006; Thomas et al., 2016b). Stress at parturition may also contribute to Hpt production by mammary tissue (Thomas et al., 2016b). The highest median Hpt concentration (5.5 g/mL, IQR: 3.1 to 9 g/mL) was observed on day 3 of lactation, and 44% of quarters showed a peak in Hpt at day 3. A similar pattern was previously observed in several cows, although median Hpt concentration decreased from day 1 to day 10 post-partum in multiparous animals (Thomas et al., 2016b).

Geometric mean SCC was higher at calving than at days 3 and 5 of lactation (Table 1), in agreement with other studies (Barkema et al., 1999; Sargeant et al., 2001). Haptoglobin concentration was significantly correlated with SCC overall ($r_s = 0.35$; $P < 0.001$; $n = 286$), at calving ($r_s = 0.21$; $P < 0.05$; $n = 94$; Fig. 1) and on day 3 of lactation ($r_s = 0.68$; $P < 0.001$; $n = 96$), but not on day 5, $r_s = 0.18$; $P > 0.05$; $n = 96$ (Supplementary Fig. 1). A positive correlation between Hpt and SCC has been described before, and neutrophils are a recognized source of extra-hepatic Hpt in milk (kerstedt et al., 2007; Lai et al., 2009). Despite the positive correlation between Hpt and SCC overall, median Hpt concentration and geometric mean SCC peaked on different days.

Table 1
Quarter-level haptoglobin concentration, somatic cell count and microbiology results of 24 dairy heifers ($n = 96$ quarters).

	Day in milk		
	1 (at calving)	3	5
Haptoglobin (g/mL)			
Positive samples[b]	76 (79%)	95 (99%)	72 (75%)
Mean[a]	7.7 1.2	8.2 1	5.6 0.8
Geometric mean	3.4	5.8	2.7
Q1	1.9	3.1	1.1
Median	3.5	5.5	3
Q3	7.1	9	7
IQR	5.2	5.8	5.9
Somatic cell count (1000 cells/mL)			
LnSCC mean[a]	6 0.3	5.5 0.1	4.8 0.1
Geometric mean[a]	381	255	124
Q1	112	90	58
Median	721	200	85 ‡
Q3	1706	558	197
IQR	1594	468	139
Microbiology[b]			
Culture-negative	39 (41%)		
< 1000 CFU/mL	35 (36%)		
1000 CFU/mL	19 (20%)		
Contaminated	3 (3%)		

Q1 first quartile; Q3 third quartile; IQR interquartile range.
Significant differences with day of calving (Friedman test, post hoc Dunn-Bonferroni test) $P < 0.01$; $P < 0.001$; with day 3 on milk $P < 0.001$.
[a] Values are mean standard error of mean.
[b] Number of quarters (% of quarters) with detectable levels of haptoglobin.

Table 2
Milk haptoglobin concentration (g/mL) in relation to culture results ($n = 93$ quarters).

	Day in milk		
	1 (at calving)	3	5
Culture-negative ($n = 39$)[c,a]	30 (77%)	39 (100%)	27 (69%)
	3 (1.9 to 5.8)	4.6 (3.2 to 6.8)	2.7 (0.4 to 5.2)
Culture-positive ($n = 54$)[c]	43 (78%)	53 (98%)	42 (78%)
Infected			
Lenient definition ($n = 54$)[a]	5.2 (1.9 to 11.1)	6.7 (3 to 10.3)	4.1 (2.1 to 8.4)
Strict definition ($n = 19$)[a]	2.3 (1.1 to 21.9)	8.1 (4.5 to 14.2)	6.3 (3.6 to 8.6)
Pure culture			
Minor pathogens			
CNS ($n = 18$)[a]	4.8 (0.4 to 15.4)	7.5 (4 to 10.3)	5.4 (2.1 to 13.8)
Corynebacterium sp. ($n = 1$)[a,b]	10	4.5	9.3
Major pathogens			
Streptococcus uberis ($n = 1$)[a,b]	2.3	49.3	8.4
Staphylococcus aureus ($n = 1$)[a,b]	5.4	6.1	4.3
Mixed culture			
CNS ($n = 6$)[a]	12.4 (1.9 to 32.8)	7.3 (4.3 to 12.5)	8.6 (4.2 to 35.8)

Significant differences with day of calving (Friedman test, post hoc Dunn-Bonferroni test) $P < 0.05$; $P < 0.01$; with day 3 on milk $P < 0.01$.
[a] Median g/mL (Q1 to Q3); ; Q1 first quartile; Q3 third quartile.
[b] Haptoglobin value.
[c] Number of quarters (% of quarters) with detectable haptoglobin.

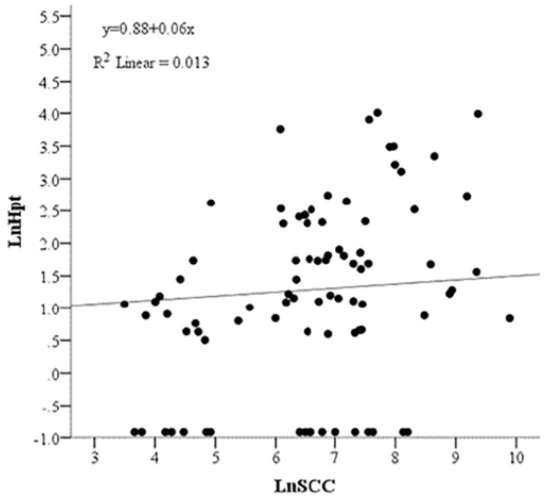


Fig. 1. Correlation between haptoglobin concentration and somatic cell count in milk from heifers on the day of calving (n = 94 quarters of 24 heifers). Linear regression equation and R^2 (coefficient of determination) value are shown.

The correlation between Hpt concentration and SCC was strongest on day 3, when all but one samples tested positive for Hpt (Table 1).

Individual quarter milk samples collected by the farmer at calving were culture negative for 41% of quarters (n = 39), culture positive with fewer than 1000 CFU/mL for 36% (n = 35), and culture positive with 1000 CFU/mL or more for 20% of quarters (n = 19). Only 3% of samples (n = 3) were contaminated, as indicated by growth of 3 or more bacterial morphotypes. This bodes well for the feasibility of on-farm or cow-side collection of high-quality milk samples for diagnostic testing.

On each sampling day, median milk Hpt concentration was greater for infected quarters than for culture-negative quarters, regardless of the definition of infection (Fig.2). Differences were not significant for

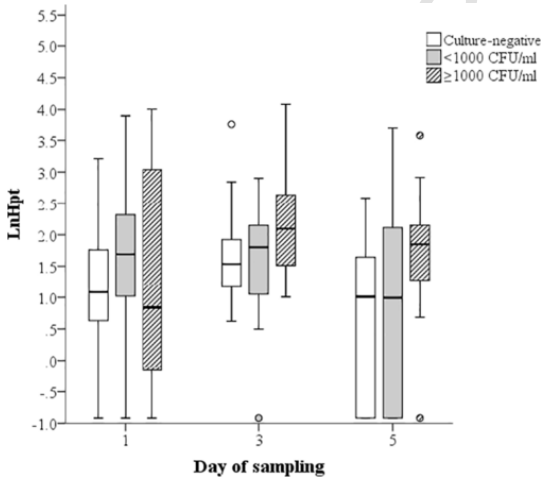
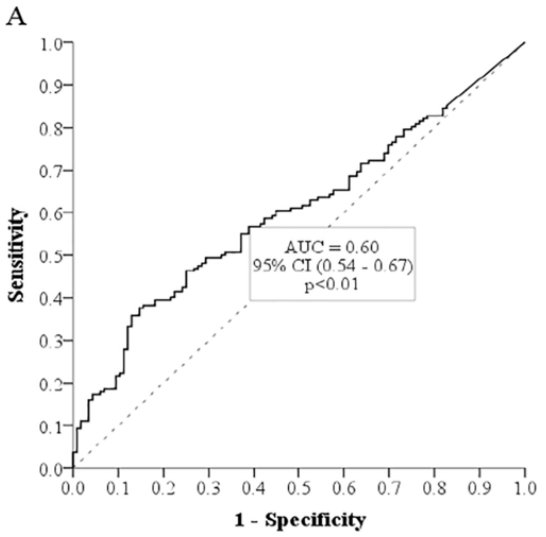


Fig. 2. Concentration of haptoglobin (natural logarithm transformed LnHpt) in colostrum (day of calving) and milk (day 3 and 5) in quarters that were culture-negative (n = 39), with low CFU count (< 1000 CFU/mL; n = 35) and high CFU count (1000 CFU/mL; n = 19) at calving. The plots show the median (line within box), 1st and 3rd quartiles (box), range (whiskers) and outliners (dots).



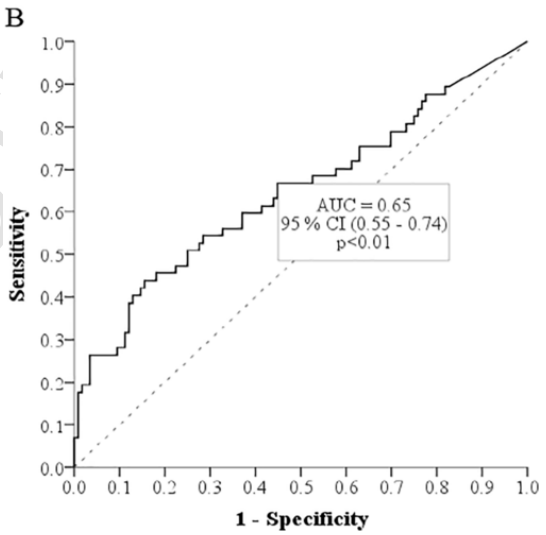


Fig. 3. Receiver operating characteristic (ROC) curves of milk haptoglobin (Hpt) for detection of intramammary infection (IMI) in dairy heifers. A; Gland status based on lenient definition of IMI (culture-positive in a non-contaminated milk sample). B; Gland status based on strict definition of IMI (1000 CFU/mL). AUC area under the ROC curve; 95% CI 95% confidence interval.

the lenient definition of infection (n = 54 or 56% of quarters). For the stringent definition of infection (n = 19 or 20% of quarters), the difference with culture negative quarters was significant on day 3 and 5 ($P < 0.05$ and $P < 0.01$, respectively; Supplementary Fig. 2). In the absence of a gold standard for IMI, it is difficult to know whether these results imply that Hpt does not accurately detect IMI with low CFU counts (false negative Hpt result), or whether low CFU counts are not truly indicative of IMI (false positive culture result).

Coagulase-negative staphylococci (CNS) were the predominant group of bacteria isolated from culture-positive quarters (89%, n = 24), of which 6 presented with 2 bacterial morphotypes (Table 2).

A predominance of CNS in samples from dairy heifers collected in the early postpartum period has been described before (Piepers et al., 2010). Haptoglobin results for different culture results are presented in Table 2. There were no statistically significant differences between Hpt concentration in pure and mixed cultures of CNS. Statistical tests to compare Hpt concentration between culture-positive quarters with minor and major pathogens were not conducted because of the small number of major pathogens isolated (*S. uberis* and *S. aureus* in one quarter each). The *S. aureus* positive quarter presented with low concentration of Hpt, similar to quarters infected with CNS in our study, regardless of day post-calving (Table 2). The presence of *S. aureus* without evidence of inflammation has been described before, and the ability to elicit an inflammatory response is strain dependent (Zadoks et al., 2000). A high Hpt concentration was observed in the quarter that was infected with *S. uberis*, particularly on day 3 post calving. After experimental challenge with *S. uberis*, Hpt levels take at least 36 h to increase (Thomas et al., 2016a). Thus, the low Hpt level at calving may indicate that the *S. uberis* infection had been acquired very recently. Considering that only two quarters were identified with major pathogens, a larger scale study would be needed to gain better knowledge of the relationship between such infections and Hpt levels in heifers post-partum.

The area under the ROC curve (AUC) of milk Hpt, 0.60 and 0.65, respectively, for the lenient and strict definition (Fig. 3) of IMI indicated a low accuracy of Hpt as a diagnostic biomarker (Swets, 1988). The cut-off value established for gland status based on lenient and strict definitions was 4.46 g/mL or 4.17 g/mL, respectively (sensitivity = 57%, specificity = 61%; or sensitivity = 60%, specificity = 63%, respectively; Fig. 3). The low sensitivity and specificity of the Hpt threshold values confirm the poor performance of Hpt as diagnostic test in heifers at calving, despite the existence of significant associations with SCC and IMI status. These results may be related with the bacterial type predominantly isolated in our samples, i.e. minor pathogens, which generally induce only mild inflammation only (Py r l and Taponen, 2009).

4. Conclusions

The data shows limited potential of milk haptoglobin as diagnostic tests for CNS intramammary infection in heifers during the first week postpartum.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rvsc.2017.05.024.

Conflict of interest statement

The authors declare no conflicts of interest. None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors thank the herdsman for his help during the study; Funmilola Clara Thomas and Andr Santana for help with the ELISA procedures; and Libby Graham, Manuel Fuentes and Kathleen Reynolds for laboratory and logistic support. Financial support from the School of Veterinary Medicine University of Glasgow is gratefully acknowledged.

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Appendix 2 – On-farm recording sheet

Thermography study - Heifers 1st thermography

Date	Shed humidity (%)	Shed temperature (C)	ε	Heifer ID	Projection	Photo (eartag, udder) (Nº)	Distance (cm)	Camera location (Nº)	Image file number	Rectal temperature	Udder palpation	Physical exam
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
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					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							
					Cd-Cr							
					Vt-Dr							

Appendix 3 – IRT temperature by bacteriological status and somatic cell count at first milking (detailed data)

Medians of $dT_{'max'}$ temperatures of the quarters in both IRT projections and sessions are presented in Table 1.

Table 1 – Maximum temperature gradient ($dT_{'max'}$) of quarters by culture status, bacterial type isolated and SCC1 category at 1st and 2nd IRT.

Udder region	Projection		Groups	1st IRT *	2nd IRT *
Quarter	Cd-Cr	*Culture Status*	Culture-negative	25.5 (24.6 – 27.6)	26.6 (23.6 – 28.2)
			<10 CFU/plate	26.1 (24.4 – 28.5)	25.1 (21.8 – 27.0)
			IMI	25.2 (23.8 – 27.1)	25.4 (21.3 – 27.0)
		Bacterial type isolated	Major pathogen	24.5 (22.8 – 24.7)	27.0 (25.7 – 27.6)
			Minor pathogen	25.0 (23.5 – 27.0)	25.4 (21.4 – 27.3)
		SCC1 categories	Very Low	25.6 (24.6 – 27.6)	24.8 (21.8 – 28.3)
			Low	26.8 (24.4 – 30.7)	25.5 (21.7 – 26.9)
			Moderate	26.3 (24.4 – 28.9)	26.4 (25.1 – 27.4)
			High	25.3 (24.4 – 28.5)	26.4 (24.1 – 27.6)
	Vt-Dr	*Culture Status*	Culture-negative	25.7 (24.9 – 28.5)	27.2 (24.0 – 28.5)
			<10 CFU/plate	26.3 (24.6 - 30.0)	26.0 (22.9 – 27.9)
			IMI	25.2 (24.4 – 26.7)	26.5 (20.8 – 28.6)
		Bacterial type isolated	Major pathogen	23.2 (23.2 – 24.1)	28.9 (26.5 – 29.2)
			Minor pathogen	25.2 (24.3 – 26.7)	26.3 (21.2 – 28.5)
		SCC1 categories	Very Low	25.8 (24.6 – 27.3)	25.8 (22.3 – 28.4)
			Low	26.4 (24.7 - 29.5)	25.9 (22.0 – 28.0)
			Moderate	25.7 (24.2 – 27.9)	27.2 (25.6 – 28.5)
			High	25.0 (23.9 -27.0)	27.1 (25.0 – 28.7)

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; *Median $dT_{'max'}$ (third quartile – first quartile); CFU – colony-forming unit.

Statistically there were no significant differences between $dT_{'max'}$ temperatures of culture status groups at first and second IRT, either at the caudocranial or ventrodorsal projections (p = 0.241 and p = 0.065 for first and second IRT craniocaudal projections, respectively; p = 0.312 and p = 0.258 for first and second IRT ventrodorsal projections, respectively).

Quarters with IMI caused by major pathogens presented lower $dT_{'max'}$ medians at first IRT than quarters with minor pathogen IMI or culture negative at

caudocranial and ventrodorsal projections (Table 1). However, those differences were significant only at the ventrodorsal projection, as shown by the low *p*-values ($p = 0.002$ and $p = 0.007$ for differences between culture-negative and major pathogen, and minor and major pathogens, respectively). Quarters infected with minor pathogens had also lower median temperatures than culture-negative quarters in both projections, but those differences were not statistically significant ($p > 0.05$). At second IRT, quarters presented higher median temperatures than in the first IRT, but there were no confirmed differences between groups for either projections, $H(2) = 3.63$, $p = 0.163$ and $H(2) = 3.19$, $p = 0.203$ for caudocranial and ventrodorsal projections, respectively.

At first IRT there were no differences between $dT_{'max'}$ of quarters included in the four SCC1 categories at the craudocranial projection, $H(3) = 6.31$, $p = 0.098$. In contrast with the ventrodorsal projection that allowed to detect overall differences of $dT_{'max'}$ between levels of SCC1, $H(3) = 11.49$, $p = 0.009$. Those differences were found between very low and low LnSCC1 quarters ($p = 0.003$); and between low and high LnSCC1 quarters ($p = 0.005$). The second IRT presented similar results, with craniodorsal projection without overall statistically significant differences ($H(3) = 4.09$, $p = 0.252$) and ventrodorsal projection showing significant differences between groups ($H(3) = 13.75$, $p = 0.003$), namely between quarters with low and moderate LnSCC1 ($p = 0.004$), and between low and high LnSCC1 ($p = 0.001$).

Medians of $dT_{'max'}$ temperatures of the teats and teat orifices in both IRT projections and sessions are presented in Table 2.

Table 2 - Maximum temperature gradient ($dT_{'max'}$) of teats and spot temperature ($dT_{'spot'}$) of teat orifice by culture status, bacterial type isolated and SCC1 category at 1st and 2nd IRT.

Udder region	Projection		Groups	1st IRT *	2nd IRT *
Teats	Cd-Cr	*Culture Status*	Culture-negative	21.5 (19.9 – 23.3)	23.5 (20.2 – 25.9)
			<10 CFU/plate	21.2 (19.8 – 22.7)	21.9 (19.3 – 25.0)
			IMI	21.1 (19.7 – 22.5)	22.0 (17.5 – 25.6)
		Bacterial type isolated	Major pathogen	19.6 (17.9 – 20.8)	24.4 (20.3 – 26.4)
			Minor pathogen	21.1 (19.7 – 22.3)	22.2 (17.7 – 25.7)
		SCC1 categories	Very Low	20.9 (19.7 – 22.7)	20.4 (18.9 – 25.5)
			Low	21.5 (20.1 – 23.8)	22.0 (18.7 – 24.1)
			Moderate	21.3 (19.8 – 23.1)	24.0 (20.8 – 25.7)
			High	21.1 (19.5 – 22.1)	23.3 (20.9 – 25.5)
	Vt-Dr	*Culture Status*	Culture-negative	23.6 (22.2 – 25.4)	24.3 (21.4 – 27.3)
			<10 CFU/plate	23.6 (22.3 – 25.5)	22.9 (20.5 – 25.9)
			IMI	22.7 (21.7 – 24.2)	22.0 (18.2 – 25.9)
		Bacterial type isolated	Major pathogen	22.2 (21.9 – 22.3)	25.6 (23.0 – 27.6)
			Minor pathogen	22.7 (21.6 – 24.2)	23.1 (18.7 – 26.5)
		SCC1 categories	Very Low	23.0 (21.9 – 24.7)	22.0 (20.3 – 25.9)
			Low	23.8 (22.6 – 25.3)	23.0 (19.7 – 25.7)
			Moderate	23.4 (22.1 – 25.5)	24.5 (22.4 – 26.6)
			High	23.2 (21.9 – 24.7)	24.5 (21.8 – 26.6)
Teat orifice	Vt-Dr	*Culture Status*	Culture-negative	15.6 (13.2 – 18.1)	19.2 (15.7 – 22.4)
			<10 CFU/plate	15.8 (10.8 – 17.7)	18.2 (16.0 – 21.2)
			IMI	14.4 (11.7 - 17.7)	18.3 (13.5 – 21.9)
		Bacterial type isolated	Major pathogen	15.6 (12.4 – 17.6)	19.9 (13.8 – 23.1)
			Minor pathogen	14.5 (12.1 – 17.9)	18.6 (13.9 – 21.9)
		SCC1 categories	Very Low	13.7 (11.3 – 17.2)	17.3 (14.7 – 21.9)
			Low	16.3 (10.7 – 18.2)	18.7 (15.3 – 21.9)
			Moderate	15.2 (12.3 – 17.9)	19.4 (16.5 – 22.5)
			High	15.6 (13.3 – 17.4)	18.8 (16.1 – 21.2)

Cd-Cr – Caudocranial; Vt-Dr – Ventrodorsal; *Median dT (third quartile – first quartile); CFU – colony-forming unit.

Teats' median values of $dT_{'max'}$ for culture status did not show significant differences between groups at 1st IRT for caudocranial and ventrodorsal projections ($H(2) = 1.06$, $p = 0.590$ and $H(2) = 2.33$, $p = 0.312$, respectively), nor at 2nd IRT for both of the projections ($H(2) = 3.41$, $p = 0.181$ and $H(2) = 3.16$, $p = 0.206$ for caudocranial and ventrodorsal projections, respectively). $dT_{'spot'}$ medians of teat orifices also did not point statistically significant differences between cultures status groups, $H(2) = 2.28$, $p = 0.319$.

The analysis of the temperature gradients by bacterial type isolated showed differences between groups on teats $dT_{'max'}$ at 1st IRT ventrodorsal projection ($H(2) = 6.92$, $p = 0.032$), but not at caudocranial projection ($H(2) = 5.93$, $p = 0.052$) or teat orifices $dT_{'spot'}$ ($H(2) = 0.07$, $p = 0.964$). The groups differing at ventrodorsal projection were the culture-negative and minor pathogen groups ($p = 0.05$), and the former from the major pathogen group ($p = 0.031$). At the second IRT set there were no differences between groups at any projection. Results of significance testing at 2nd IRT for teats $dT_{'max'}$ caudocranial projection were $H(2) = 1.64$, $p = 0.440$; for teats $dT_{'max'}$ ventrodorsal projection $H(2) = 2.81$, $p = 0.245$; and for $dT_{'spot'}$ ventrodorsal projection $H(2) = 0.69$, $p = 0.709$.

Teat's $dT_{'max'}$ medians did not differ between levels of LnSCC1 at 1st and 2nd IRT for the craniocaudal projection ($H(3) = 4.73$, $p = 0.192$ and $H(3) = 5.76$, $p = 0.124$ for 1st and 2nd IRT, respectively). However, at the ventrodorsal projection there were differences between groups at both occasions of IRT data collection ($H(3) = 10.54$, $p = 0.015$ and $H(3) = 14.34$, $p = 0.002$ for 1st and 2nd IRT, respectively). At the 1st IRT, the post hoc test identified statistically significant differences between very low and low LnSCC1 groups ($p = 0.002$), but not between the other groups. At 2nd IRT there was a significant difference between the low and high LnSCC1 groups ($p = 0.0005$). $dT_{'spot'}$ medians for teat orifices did not differ between groups at 1st IRT ($H(3) = 7.04$, $p = 0.071$) neither at 2nd IRT ($H(3) = 4.94$, $p = 0.177$).

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