Tools for targeted treatment of bovine clinical mastitis

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Summary

Mastitis is the most common and expensive health problem of dairy cattle. Its control and management are the main reasons for antimicrobial usage (**AMU**) in dairy cattle with the potential for the development of antimicrobial resistance (**AMR**), as introduced in **Chapter 1**. To limit AMU, farmers are increasingly encouraged to adopt targeted treatment decisions based on knowledge of the pathogens causing clinical mastitis (**CM**), whereby treatment of non-severe CM is generally recommended for gram-positive mastitis but not for gram-negative or culture-negative mastitis. In **Chapter 2** the current knowledge of mastitis pathogens and their management is discussed with a specific focus on treatment, and host, farm or pathogen factors that influence the probability of treatment success. Such knowledge, combined with the use of on-farm diagnostics, could contribute to reduced AMU for mastitis control. In addition, a review of currently available on-farm diagnostics is provided. This is followed by original research to investigate the performance of different tools to support targeted treatment based on the differentiation of gram-positive CM from other cases of CM using two different approaches.

In the first approach, presented in **Chapter 3** the aims were to conduct a laboratory-based evaluation of the performance of a simplified slide test as a tool to differentiate gram-positive CM from other cases of CM, and to compare its performance against a commercially available on-farm test that is commonly used in my area (VétoRapid). Test outcomes were compared to results from bacteriological culture and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Milk samples (n = 156) were obtained from cases of severe and non-severe CM on seven farms and collected by farm personnel. After removal of contaminated samples (n = 23) and organisms with unknown species identity (n = 3), the simplified slide test showed high sensitivity and accuracy (> 80%), similar to the comparator test. For most outcomes of interest (culture positive, *Escherichia coli*, or gram-positive growth), the specificity of the slide test (85.7, 97.5 and 76.8% respectively) was higher than the specificity of the comparator test (57.1, 92.4 and 58% respectively). When considering non-severe cases of CM only and interpreting detection of gram-positive organisms as an indicator of the need for antimicrobial treatment, the simplified test had higher specificity than

the comparator (77.4% vs 60.4%) and similar sensitivity (83.9% vs 87.5%). The proportion of sampled CM cases, contaminated samples and gram-positive mastitis cases differed between farms, which affects the positive and negative predictive value, the economic value of diagnostic testing and its potential to reduce AMU.

Culture-based tests have the inherent limitation of long turn-around time. Culture-independent tests based on biomarkers of inflammation are used as a proxy for infection. If results of biomarker tests were pathogen-specific they could potentially be used to inform targeted treatment decisions with shorter turn-around times than culture. Therefore, a second approach presented in **Chapter 4**, investigated if a recognised mastitis biomarker could be used to differentiate mild-to-moderate gram-positive CM from other forms of CM. To detect cathelicidin presence two Western blot techniques (A and B) were performed using CM samples and healthy quarter milk samples obtained by farm personnel. As for the first approach, outcomes were compared to results from bacteriological culture and MALDI-ToF MS. To detect gram-positive bacteria technique A showed variable sensitivity (31.6-68.4%) and specificity (55.6-81.5%), with moderate accuracies (< 67.4%). Agreement between gels was slight (κ = 0.109) and moderate (κ = 0.572) using exposures of 30 seconds and 5 minutes, respectively, demonstrating poor repeatability of technique A. Technique B showed variable sensitivity (37.5-87.5%), low specificity (15.4-38.5%) and moderate accuracies (< 73.5%). The agreement between gels was almost perfect (κ = 0.9) and perfect (κ = 1) using exposures of 5 minutes and 30 seconds, respectively, showing high repeatability of this method. When considering non-severe cases of CM and interpreting detection of cathelicidin presence as an indicator of the need for antimicrobial treatment, i.e. as a marker of gram-positive mastitis, the biomarker had moderate-high sensitivity (75%), low specificity (30%) and low accuracy (42.9%) using the technique B. In healthy quarter milk samples, cathelicidin was not detected (n = 9). This approach showed that the biomarker failed to support targeted treatment decisions based on differentiation of gram-positive CM but supports its use as a general mastitis biomarker. Refinement of cathelicidin testing through detection of cathelicidin subtypes may improve its usefulness as indicator for gram-positive mastitis and treatment. Farmer needs, attitudes and behaviour are crucial for on-farm test uptake, as discussed in the final **Chapter**. Farmers want a quick and simple test to inform CM treatment decisions. The simplicity of the slide test could

make it an attractive tool to target antimicrobial treatment of non-severe clinical mastitis with good accuracy. The short turn-around time of biomarker-based tests, allowing farmers to generate results and make treatment decisions between two rounds of milking, warrants further work on the evaluation of refined cathelicidin testing.

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Peer reviewed journals

Malcata, F.B., P.T. Pepler, R.N. Zadoks, L. Viora. 2021. Laboratory-based evaluation of a simplified point-of-care test intended to support treatment decisions in non-severe bovine clinical mastitis. Journal of Dairy Research, 88(2), pp. 170-175 (see **Appendix 1**)

Malcata, F.B., P.T. Pepler, E.L. O'Reilly, N. Brady, P.D. Eckersall, R.N. Zadoks, L. Viora. 2020. Point-of-care tests for bovine clinical mastitis: What do we have and what do we need? Journal of Dairy Research, 87, pp. 60-66 (see **Appendix 2**)

Conferences

Malcata, F.B., P.T. Pepler, L. Viora, R.N. Zadoks 2019. Comparison of two point-of-care tests to support treatment decisions in non-severe bovine clinical mastitis. Poster and oral presentation, British mastitis conference 2019, November 2019, Worcester, England, UK

Malcata, F.B., P.T. Pepler, L. Viora, R.N. Zadoks. 2019. Can point-of-care tests for bovine mastitis reduce antimicrobial usage without animal welfare loss? Oral presentation, European Bovine Congress 2019, "Your veterinary toolbox 2025", September 2019, 's-Hertogenbosch, The Netherlands

Malcata, F.B., P.T. Pepler, L. Viora, R.N. Zadoks. 2018. Evaluation of a selective culture-based media for detection and differentiation of bacteria associated with bovine clinical mastitis. Poster presentation, British mastitis conference 2018, November 2018, Worcester, England, UK

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Author's declaration

I, Francisco Malcata, declare that the work in this thesis is original, and unless stated below as work done by others and in all the Material and Methods sections, it was my own work with due acknowledgements. This work has not been submitted for any other degree or professional qualification.

The work done by others:

- Research ethics application (Lorenzo Viora)
- Project concept (Lorenzo Viora and Ruth Zadoks)
- On-farm sample collection (Farm staff)
- Isolates speciation through MALDI-ToF MS (external laboratories)
- Statistical analysis (calculation of the significance of differences between tests) (Theo Pepler) and Pairwise t-test analysis (using Bonferroni-error correction to adjust for multiple comparisons) (Laurianne Schoenberger)

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Definitions/abbreviations

AMR	Antimicrobial resistance
AMU	Antimicrobial usage
BCA	Bicinchoninic acid
CM	Clinical mastitis
DIM	Days in milk
DCT	Dry cow treatment
HP-CIA	Highest-Priority Critically Important Antimicrobials
IMI	Intramammary infection
IMM	Intramammary
ITS	Internal teat sealant
LAMP	loop-mediated isothermal amplification
LPS	Lipopolysaccharides
MCB	Mastitis causing bacteria
NAS	Non-aureus staphylococci
NSAID	non-steroidal anti-inflammatory drug
PCR	Polymerase chain reaction
RPM	Rotations per minute
SBA	Sheep blood agar
SCC	Somatic cell count
SCM	Subclinical mastitis
US	United States
UK	United Kingdom
WHO	World Health Organization

Chapter 1. General Introduction

Food demand is estimated to increase by half between 2012 and 2050. This demand is a result of population growth, increased urbanization, and per capita increase in incomes (FAO, 2017). At the same time, we are living in an environment where natural constraints, such as natural resource degradation and climate change are of growing concern and putting the limited natural resources under increased pressure. However, satisfying these extra demands with the farming practices of today is likely to lead to higher competition for natural resources, increase greenhouse gas emissions, increase deforestation, and land degradation. There is a critical need to stop and reverse these environmental issues, allowing at the same time to produce food efficiently to meet the world needs (FAO, 2017). To respond to this increase in demand and address these constraints "we need to produce more with less" (Foresight, 2011). Technological advances allow better use of resources and higher production of extra outputs with an improvement in food safety.

Mastitis, an inflammation of the mammary gland mostly caused by bacterial infection, is the most common and expensive health problem of dairy cattle (Ruegg, 2003). The presence of mastitis in dairy herds is an example of production inefficiency (Halasa *et al.*, 2007), and controlling mastitis is an important way to "produce more with less". Also, mastitis can be a painful disease and may affect cow welfare (Leslie and Petersson-Wolfe, 2012). Finally, mastitis control and management is also the main reason for antimicrobial usage (**AMU**) in dairy cattle (Pol and Ruegg, 2007b), with several consequences, including discarded milk (Ruegg, 2003), presence of antimicrobial residues in milk (Garcia *et al.*, 2019) and potential for the development of antimicrobial resistance (**AMR**) (Pol and Ruegg, 2007a; Oliver and Murinda, 2012).

AMR is currently a critical public health issue. Today, approximately 700 000 humans die per year as a result of infections caused by multi-resistant bacteria (O'Neill, 2014). The predictions suggest that by 2050 around 10 million people will die from bacterial infections caused by multi-resistant bacteria if no actions are taken to reverse behaviours that are leading to an increase in AMR prevalence (O'Neill, 2014). This constitutes a worldwide problem, and

the World Health Organization (**WHO**) is already taking specific initiatives to prevent AMR (WHO, 2015). WHO identified priority pathogens that need extra caution and new strategies to control them, as well as antimicrobials that are considered of critical importance in human medicine (WHO, 2017). In 2019, WHO identified Highest-Priority Critically Important Antimicrobials (**HP-CIA**) for human medicine and discouraged usage of HP-CIA in livestock production (WHO, 2019; Responsible Use of Medicines in Agriculture Alliance (RUMA), 2020). Examples of priority pathogens are *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus* and HP-CIA include quinolones, macrolides and ketolides, polymyxins and 3rd and 4th generation cephalosporins. Those pathogens and antimicrobials have all been linked with bovine mastitis (Klaas and Zadoks, 2017).

Veterinarians have the role to preserve bovine udder health, guarantee animal welfare and prevent economic losses. In addition, veterinarians must assume their responsibility to combat AMR emergence and preserve antimicrobial efficacy for the time to come. There is an imperative for the reduction in AMU in the dairy industry. Mastitis management relies too much on antimicrobials (Klaas and Zadoks, 2017). Regarding mastitis treatment, there is an opportunity to reduce AMU and stop using HP-CIA (Turner *et al.*, 2018; Doehring and Sundrum, 2019; Nobrega *et al.*, 2020). Societal pressure to reduce AMU in farming forces us to improve our knowledge and to develop new mastitis management approaches that promote prudent AMU (OIE, 2016). This involves appropriate prescription and avoiding inappropriate AMU to preserve antimicrobial efficacy for as long as possible (Centers for Disease Control and Prevention, 2007).

In some countries quota to reduce AMU has been implemented (Dorado-García *et al.*, 2016). In the United Kingdom (**UK**) there are no government-mandated restrictions relative to AMU in livestock production yet. However, to meet the requirement of food standard schemes dairy farmers in the UK need to undertake an annual review of AMU with their veterinarian. And from the 1st of June 2018, the use of HP-CIAs must be the last option and restricted to cases where evidence of sensitivity or diagnostic testing justifies its use (Red Tractor Assurance, 2018). Policies like these or targets for AMU set in the Netherlands (Bos *et al.*, 2015) are needed to reduce AMU and may become key drivers to implement targeted selective treatment.

The use of selective treatment in lactating and non-lactating animals, respectively, is a practice that restricts AMU to cases that are more likely than others to benefit from treatment (Lago and Godden, 2018), or to cases most likely to have an intramammary infection at dry-off (Vanhoudt *et al*., 2018). It aims to maximize treatment efficacy, animal health and welfare, while at the same time promoting prudent AMU and minimizing potential selection for AMR. This approach is often described as selective treatment or targeted treatment. "Selective treatment" recommends blanket treatment to be the default option, which is modified by selecting cows for treatment. "Targeted treatment" suggests no treatment to be the default option, with the targeting of treatment to those cows that are most likely to benefit from it (Malcata *et al.*, 2020). Selective dry cow treatment (**DCT**) is adopted in many countries (Vanhoudt *et al.*, 2018), selective treatment of clinical mastitis (**CM**) in lactating animals is still a recent practice (Lago and Godden, 2018) and requires a deep understanding of different manifestations of mastitis and its epidemiology. Ideally, this would include knowledge of severity, causative agent and prognosis, so that treatment with anti-inflammatory, antimicrobial or supportive products are adapted to each CM case. In Chapter **2**, I will review the biological aspects of mastitis and current treatment modalities and explore potential opportunities for improvements in CM treatment with less AMU. Diagnostics play an important role in such improvements.

In Chapter **3**, I describe a new diagnostic test that is based on culture, like most current assays, but which may have advantages in terms of simplicity and user-friendliness. Culture-based tests have a long turn-around time, which is a major limitation. This limitation could be overcome with the use of biomarkers, which are not culture dependent. In Chapter **4**, I explore the potential of a biomarker as a diagnostic indicator. Finally, in Chapter **5**, I discuss results from the previous chapters with an emphasis on non-technical aspects of test uptake, such as farmer attitudes.

Chapter 2. Literature review

2.1 Types of mastitis

Mastitis is an inflammation of the mammary gland, frequently caused by intramammary infections (**IMI**). Most IMI are caused by bacteria, although they can be caused by yeasts or algae. In rare cases, mastitis can be caused by mechanical or chemical trauma, e.g. through teat or udder trampling, or when cleaning liquids are mistaken for teat disinfectants. On some occasions, the cause of mastitis is not detected. These situations can be frustrating because of the difficulty to manage the disease when its aetiology is not known, or frustration about costs of diagnostic tests that yield negative results.

Mastitis is characterized by physical and chemical changes in milk and, in some cases, by pathological changes in the mammary gland tissue or at a systemic level. The magnitude of the inflammatory response and the degree of change depends on the cause of mastitis and host factors. Clinical mastitis occurs when physical changes in milk or in the quarters can be detected using human senses (vision, touch, and taste, although the latter is no longer recommended because of health and safety concerns), whereas detection of subclinical mastitis (**SCM**) requires the use of additional tools. Most cells in milk are white blood cells, normally referred to as somatic cells, and somatic cell count (**SCC**) is used as an indicator of mastitis in cows. When the milk appears physically normal as judged by the naked eye, but is high in SCC, this is considered an indicator of SCM.

A cow can develop CM immediately after an intramammary challenge or after a prolonged period of subclinical infection. After resolution of a CM episode (clinical cure), a quarter can return to the uninfected state or to a state of SCM and persistent IMI, which can flare up again in CM later on (Bradley and Green, 2001; Zadoks *et al.*, 2003). There are three clinical forms of CM. In mild CM, only altered milk is detected (Wenz *et al.*, 2001; Pinzón-Sánchez and Ruegg, 2011). These represent between 40% and 50% of CM cases (Oliveira *et al.* Ruegg, 2013; Levison *et al.*, 2016). Moderate CM is characterized by abnormalities in milk and udder quarter (e.g. heat, pain, swelling or redness). Severe CM cases are characterized by

abnormal milk, with abnormalities of the gland, and with the cow presenting systemic signs of illness (e.g. fever, tachycardia, tachypnoea, dehydration, decreased ruminal function or anorexia) (Wenz *et al.*, 2001; Lago *et al.*, 2011; Pinzón-Sánchez and Ruegg, 2011). IMI can occur in the dry period or during lactation and may manifest as SCM or CM in either phase of the lactation cycle.

In the past, based on pathophysiology and epidemiology, mastitis pathogens have been classified as contagious, environmental or teat skin opportunists (Radostits *et al.*, 2007). Contagious bacteria primarily exist within the mammary gland and are transmitted from cow to cow during the milking process. They are associated with persistent infections normally reflected by a raised SCC. This type of mastitis was a major problem in the past, however standard prevention programmes allowed for a reduction in their prevalence (Royster and Wagner, 2015). The pathogen distribution varies between countries, but *Staph. aureus*, *Mycoplasma* spp. and *Streptococcus agalactiae* are normally important contagious bacteria (Royster and Wagner, 2015), whereas *Streptococcus dysgalactiae* and *Streptococcus uberis* can be contagious as well as environmental, depending on herd management and environmental conditions. Environmental mastitis is caused by pathogens derived from the environment. They can be acquired during milking, between milkings, or without milking (e.g. by heifers and dry cows). The bacteria most likely to infect cows from the environment include coliforms and *S. uberis*. The cause of mastitis, particularly when caused by IMI, can rarely be detected without further testing. Even severe CM, often called “toxic mastitis” and attributed to *E. coli*, can be caused by *S. uberis* or *Staph. aureus* (Zadoks *et al.*, 2000; Tassi *et al.*, 2013). Conversely, coliform species can be responsible for moderate or mild CM or even SCM (Bradley and Green, 2000). Mild-to-moderate forms of CM can also be caused by *Strep. agalactiae* (Simões Cortinhas *et al.*, 2016). Thus, there is no absolute rule regarding different causative agents of mastitis and clinical severity.

The most common outcomes when culturing milk from CM are no growth, *E. coli* and “environmental streptococci” (Bradley *et al.*, 2007; Oliveira *et al.*, 2013). The latter, however, should be referred to as “gram-positive, catalase-negative cocci”, as the genus of the organism is often not determined, and this category includes Streptococcus, Enterococcus, Lactococcus,

and other genera (Klaas and Zadoks, 2017). Opportunistic mastitis pathogens are normally commensal bacteria from the teat skin that can cause mastitis after entering the udder through the streak canal. Non-aureus Staphylococci (**NAS)** are the most common bacteria from this group. Today, we know that the distinction between contagious and environmental pathogens at the species level may be misleading, instead, it should be applied at the strain level (Zadoks *et al.*, 2011). Correct differentiation of epidemiology and modes of transmission as contagious or environmental pathogens would allow the implementation of mastitis control methods tailored to the farm's situation (Klaas and Zadoks, 2017).

Other type of mastitis, with distinct characteristics are Summer mastitis. This form of mastitis affects essentially dry cows and heifers in pasture, but it can also affect calves and bulls. This type of mastitis occurs mostly in Summer months and in temperate climates of North Hemisphere. In England and Wales, it has been reported to affect 39-54% of the herds with 2.1 to 4.1 cases per affected herd (Berry, 1998). Several pathogens have been associated to this type of mastitis, such as *Truepurella pyogenes*, *Peptococcus indolicus*, *Streptococcus dysgalactiae*, *Fusobacterium necrophorum*. In acute phase animals with Summer mastitis have pyrexia, anorexia, with enlarged and swollen udder and teat, that may cause discomfort and altered mobility score. A watery secretion with clots may be recovered if the quarter is stripped. In some cases, animals may develop septicaemia or toxaemia. In severe cases, the quarter can become gangrenous or an abscess may develop, that can burst at the surface of the udder (Ahmad *et al.*, 2015; Blowey and Edmondson, 2010). One of the most important risk factor for this type of mastitis is the seasonal activity of the sheep head fly, *Hydrotea irritans*, a non-biting fly, that feeds on secretions, and is known as a vector for transmission of the main bacteria *T. pyogenes* in the teat end. Other important risk factors are trauma or damage of the teat due to poor milk settings, the presence of spiky plants on pasture that can cause irritation to the udder skin and cross sucking among animals (Blowey and Edmondson, 2010).

2.2 Mastitis pathogens

Mastitis-causing pathogens can be classified in major and minor pathogens according to their severity, virulence, and capacity to damage the udder. The most common major mastitis pathogens are *Strep. agalactia*e, *S. uberis*, *Strep. dysgalactiae*, *Staph. aureus, Mycoplasma* spp. and gram-negative coliforms, such as *E. coli* and *Klebsiella* spp. By contrast, *Corynebacterium bovis* and NAS are considered minor pathogens as they lack the ability to cause severe CM (Schepers *et al.*, 1997; Reyher *et al.*, 2012a), and their pathogenicity is limited to moderate increase in the SCC, up to mild CM, in rare occasions (Djabri *et al.,* 2002; Pyörälä and Taponen, 2009a).

This distinction between major and minor pathogens is particularly important because SCM and mild CM caused by NAS may be left untreated (Pyörälä and Taponen, 2009a), as spontaneous cure rates are around 70% (McDougall, 1998; Wilson *et al.*, 1999).

2.2.1 Major Pathogens

2.2.1.1 Gram-negative coliforms

Coliform mastitis has been commonly associated with "environmental mastitis" and in practice, there is the perception that they are commonly responsible for severe CM cases. IMI caused by coliforms may start in the dry period as SCM and can develop into CM in early lactation (Bradley and Green, 2000). Severe CM can cause bacteraemia with mastitis pathogens or bacteria from lungs and gut origin (Wenz *et al.*, 2001).

2.2.1.1.1 Escherichia coli

Mastitis caused by *E. coli* is mostly transient with clinical presentation dependent on host factors (see **2.4.2.1** Host factors section), environmental hygiene (Klaas and Zadoks, 2017) and different strains. *E. coli* strains are generally commensals, but they can also be pathogenic. To date, the ability to cause mastitis has not been linked to specific virulence traits (Keane, 2019), supporting that *E. coli* mastitis is mostly related to host factors. Mild and moderate CM cases are normally of transient duration and they usually cure spontaneously (Pyörälä *et al.*,

1994; Roberson, 2012). Severe cases can develop bacteraemia (Suojala *et al.*, 2013). On some occasions, *E. coli* IMI can be persistent with multiple episodes of CM alternated with SCM. Clinical mastitis caused by this pathogen is responsible for a significant decrease in milk yield that may last until the end of lactation (Heikkilä *et al.*, 2018) and important negative effects on fertility, especially for severe cases (Lavon *et al.*, 2019; Dalanezi *et al.*, 2020).

2.2.1.1.2 Klebsiella spp.

Klebsiella spp. IMI causes generally more severe clinical signs than *E. coli*, is characterized by a stronger immune response, with higher lethality (Schukken *et al.*, 2012), greater risk of culling (Fuenzalida and Ruegg, 2019a), reduced rate of spontaneous cure (Pinzón-Sánchez *et al.,* 2011), and a tendency to become chronic (Fuenzalida and Ruegg, 2019b). Milk production losses due to CM caused by this genus are substantial. Multiparous cows, in particular, produced 4.9 Kg less milk per day in comparison with unaffected cows (Hertl *et al.*, 2014). Pathogenicity of *Klebsiella pneumoniae* bacteria in the udder is not well understood, but some virulence factors such as capsular serotypes, mucoviscosity-associated gene have been identified, and can explain some of the severity caused by IMI of this pathogen (Osman *et al.*, 2014).

2.2.1.2. Staphylococcus aureus

Most *Staph. aureus* behave as contagious organisms, however, some strains can be environmental (Klaas and Zadoks, 2017). Cows can become infected with their own or environmental strains of this pathogen (Zadoks *et al.*, 2011). The pathogenicity of this pathogen and its ability to colonize the skin and mucosal epithelia, and its poor response to treatment in some cases (Barkema *et al.,* 2006), make IMI caused by this pathogen a problem in the dairy industry (Rainard *et al.*, 2018). Mastitis cases caused by this pathogen are commonly SCM, manifested with increased SCC. Most of the IMI are chronic and can persist for the entire or even the following lactation, with CM flare-ups with variable severity. CM cases can manifest with a sudden rise in SCC and acute signs of pyrexia and anorexia, even before detectable changes in the milk. After these clinical signs are when alterations in the udder occur, lasting

for few days, then the infection becomes subclinical. In some IMI, it remains as SCM and can spread widely among the herd mates. But on other occasions, it can be severe CM, manifested with per acute systemic signs and ischaemic gangrene in the quarter and teat. The severity of the disease is associated with host (see **2.4.2.1** Host factors section) and pathogen factors (see **2.4.2.4** Pathogen factors section) and the spread of infections is associated with strains' contagiousness and husbandry practices (Rainard *et al.*, 2018). Chronically infected mammary glands are the main reservoir of *Staph. aureus* in herds, but it also can colonise teat and inguinal skin, nares and hocks (Capurro *et al.*, 2010; Rainard *et al.*, 2018). The bacteria are mainly spread by teat cup liners during the milking process, milkers' hands and washcloths. Infections in heifers that did not come in contact with any milking machine can be due to flies (Anderson *et al.*, 2012). Cows diagnosed with CM with this pathogen suffered a significant and long-term loss in milk production. The milk yield decrease is larger when the IMI occurred in early lactation but was similar in both CM and SCM (Heikkilä *et al.*, 2018).

There are generally multiple strains of this pathogen within a herd, often but not necessarily, with a predominance of one strain, reflecting whether its transmission is contagious or environmental (Sommerhäuser *et al.*, 2003). Strains can differ in their ability to spread, cause CM, increase SCC, cause persistent IMM or affect milk production (Barkema *et al.,* 2006).

2.2.1.3 Gram-positive catalase-negative cocci

These are commonly referred to as "environmental streptococci" or "*Streptococcus* spp.", although this is a misnomer because the group includes several bacterial genera, including streptococci, enterococci and lactococci, among other pathogens. The major pathogens of this group are *S. uberis*, *Strep. dysgalactiae* and *Strep. agalactiae*. The major enterococci are *Enterococcus faecium* and *Enterococcus faecalis*, and the main lactococci are *Lactococcus lactis* and *Lactococcus garvieae* (Klaas and Zadoks, 2018). Little is known about shedding patterns and pathogenesis of these pathogens except for *S. uberis* and *Strep. dysgalactiae*.

2.2.1.3.1 Streptococcus uberis

S. uberis is a common udder pathogen able to cause both SCM and mild to severe CM. This pathogen has the ability to cause persistent infection and recurrent clinical flare-ups

(Pedersen *et al.*, 2003; Milne *et al.*, 2005; Keane, 2019). There is some evidence of strain-associated virulence (Zadoks *et al.*, 2003; Keane, 2019). This pathogen has both contagious and environmental modes of transmission (Zadoks *et al.*, 2001; Wente *et al.*, 2019), subject to geographic variations, and in the UK, most are contagious (Davies *et al.*, 2016). It can be found in other sites than the mammary gland, e.g. the gastro-intestinal tract, soil, water, forage and insects. However, faecal shedding is needed to maintain it in the environment (Zadoks, 2007). New IMI may occur in lactating cows through the milking parlour, or from the environment and all the animals in the herd are susceptible to this form of transmission (Zadoks *et al.*, 2003). There is a different ability of *S. uberis* strains to cause disease (Tassi *et al.*, 2013, 2015).

2.2.1.3.2 Streptococcus dysgalactiae

Strep. dysgalactiae has been described as both contagious and environmental pathogen responsible for SCM and CM cases. This pathogen has been identified in the rumen, faeces, muzzle, tonsils, vagina, bedding and barn environment (Cobirka *et al.* 2020), or associated with teat lesions (Ericsson Unnerstad *et al.*, 2009). Infection rates in some countries are higher during the summer pasture season (Lundberg *et al.*, 2016), in other countries in the housing season (Olde Riekerink *et al.*, 2007). Despite being a common mastitis pathogen, few studies have focused on this pathogen.

2.2.1.3.3 Streptococcus agalactiae

Strep. agalactiae IMI is usually chronic and subclinical, with intermittent episodes of CM. Infections are characterized by an increase in SCC and a decrease in milk yield (Keefe, 1997). This pathogen is highly contagious and easily transmitted from cow to cow during milking. With infected cows acting as a reservoir of infection and contaminating milking equipment and milkers' hands (Keefe, 2012). It was considered an obligate IMM pathogen (Keefe, 1997), but it was found in environmental reservoirs, such as water throughs and stalls, or in the rectum of the cows (Jørgensen *et al.*, 2016), and is also carried by a significant proportion of people (Skov Sørensen *et al.*, 2019). Historically this pathogen has been eradicated in some herds by treating all quarters when a composite milk sample yielded this pathogen,

regardless of the number of infected quarters – "Blitz therapy" (Erskine *et al.* 2003). Implementation of mastitis control practices resulted in elimination of this bacteria from several herds is western countries, but it re-emerged recently in northern Europe, probably due to changes in herd size, housing and milking systems, as well as the emergence of new strains with new epidemiological characteristics. For example, faecal, environmental, or human reservoirs of *Strep. agalactiae* may exist in some herds (Jørgensen *et al.*, 2016; Skov Sørensen *et al.*, 2019).

2.2.1.4 Mycoplasma spp.

Mycoplasma spp. has been known as a mastitis pathogen since the 1960s in the US (Hale *et al.*, 1962) and since then it has emerged as an important pathogen worldwide (Nicholas and Ayling, 2003). Because of its difficult detection through bacterial culture and the need to use specific culture media, its prevalence is probably underestimated (De Vliegher *et al.*, 2012). *Mycoplasma bovis* is the most pathogenic species of the genus *Mycoplasma*. This organism is associated with mastitis and other diseases (Calcutt *et al.*, 2018). Infected animals can become asymptomatic carriers and shed the organism through nasal discharge and milk for months to years and diagnosing these animals can be challenging. Regarding udder health, it has been associated with SCM, CM or chronic mastitis. In acute CM cases, the mammary gland of a single quarter or multiple quarters can be severely compromised, showing a serous or purulent exudate. CM is characterized by hard quarters, with a sudden drop in milk and failure to respond to antimicrobial treatment (Calcutt *et al.*, 2018). In other cases, there are no systemic signs of disease, and clinical presentation is limited to a mucoid discharge or signs of arthritis or even abortion. *M. bovis* is very contagious and transmission is generally by aerosols. Transmission can occur at milking time from a reservoir, the infected udder; via fomites, hands of a milker, milking unit liners, or udder washcloths (Fox, 2012). Calves can also be infected by ingesting milk from infected cows or during foetal development if the dam was infected. Semen can also transmit the organism, via artificial insemination (Haapala *et al.*, 2018) or using an infected breeding bull (Gille *et al.*, 2018). Bedding can also be a source of infection (Piccinini *et al.*, 2015). It is speculated that environmental stresses, and having open herds that introduce cattle

from external herds are risk factors for outbreaks of this disease (Punyapornwithaya *et al.*, 2011; Calcutt *et al.*, 2018).

2.2.2 Minor Pathogens

IMI by minor pathogens result in minor or absent damage to the bovine mammary gland with a minor inflammation, detectable by low increase in SCC (Reyher *et al.*, 2012a). However, milk production losses by these pathogens should not be neglected (Heikkilä *et al.*, 2018). The relative importance of minor pathogens depends on the prevalence of major pathogens, and milk quality standards in the region or country (Schukken *et al.*, 2009). An important aspect is that the presence of these pathogens (NAS and corynebacteria) at the teat end may protect against infection with major pathogens (Reyher *et al.*, 2012b).

2.2.2.1 Corynebacterium bovis

Corynebacterium bovis is regarded as a minor pathogen that causes a mild increase of SCC, rarely associated with CM, and if CM occurs, cases are normally mild (Hiitiö *et al.*, 2016; Heikkilä *et al.*, 2018) and with limited decreased yield (Heikkilä *et al.*, 2018). This pathogen has been considered as a colonizer of the teat canal (Bexiga *et al.*, 2011) but was detected in similar numbers when milk samples were collected via the teat canal or directly from the udder cistern (Heikkilä *et al.*, 2018). Some authors reported that this pathogen can have an important protective role against IMI caused by other pathogens (Rainard and Poutrel, 1988), whereas other authors report an association with an increased risk of mastitis (Berry and Hillerton, 2002). Conflicting results may be due to differences between experimental studies and field observations (Reyher *et al.*, 2012b). Another possible explanation for this polarization of opinions is that the protective effects of this pathogen are probably masked because glands with IMI are innately susceptible to repeat infections irrespective of the protective effect of *Corynebacterium* spp. in IMI (Parker *et al.*, 2007).

2.2.2.2 Non-aureus staphylococci

Non-aureus staphylococci are the most frequently isolated bacteria that cause IMI, but they also colonize the teat-ends and can be detected in cows' faeces (Wuytack *et al.*, 2020). They are generally considered a group of minor pathogens that may include more than 20 different species. The most commonly detected species include *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis* and *Staphylococcus hyicus.* There are conflicting results regarding their importance, and there are variations in virulence among species and strain (Vanderhaeghen *et al.*, 2014). Some might be due to identification issues because identification of these bacteria at the group level rather than species level has been a standard procedure in both diagnostics and research (Supré *et al.*, 2011). IMI by these pathogens generally results in SCM with moderate SCC increase or mild CM. This might be related to a low bacterial load in the milk probably illustrative of the low colonization level of the mammary gland. Clinical signs differ among different NAS species and among studies, which is partly due to different study designs, species under investigation and methods for species identification (Vanderhaeghen *et al.*, 2014). In an experimental study, *Staph. simulans* infection caused higher SCC than infections by *Staph. epidermis* (Simojoki *et al.*, 2011). *Staph. simulans* and *Staph. chromogenes* caused SCM and CM in approximately equal proportions and no species was noted to be significantly different in terms of severity or clinical presentation (Taponen *et al.*, 2006). *Staph. chromogenes* was the most prevalent species in CM (Zadoks and Watts, 2009; De Visscher *et al.*, 2016; Condas *et al.*, 2017). *Staph. hyicus* has been more commonly associated with CM than SCM (Persson Waller *et al.*, 2011). An important aspect is that IMI or teat apex colonization by NAS may have a protective effect on udder health, although the protective effect varied among studies. In observational studies, the IMI with these minor pathogens had no protective effects against major pathogens. Contrary to that, in challenge studies, their protective effect was significant and particularly when major pathogens were introduced into the mammary gland via methods bypassing the teat end (Reyher *et al.*, 2012b). These effects were higher for IMI by NAS than for *C. bovis* IMI (Reyher *et al.*, 2012b). The protective effect of teat apex colonization by NAS was shown in heifers, specifically against IMI caused by major pathogens in early lactation (Piepers *et al.*, 2011). Teat apex colonization with *Staph. chromogenes* prepartum protected

quarters at the beginning of lactation from having SCC above 200.000 cells/ml, and influence the entry of major pathogens (Vliegher *et al.*, 2003) There is a knowledge gap in how these bacteria cause IMI and disease. A recent study of more than 190 genes in 25 NAS species demonstrated that suspected virulence factors are generally not associated with disease severity. The severity of mastitis caused by these bacteria is associated with an increased number of toxin that these bacteria produce and host immune evasion genes (Naushad *et al.*, 2019).

Mastitis management approaches to decrease mastitis incidence by improving measures to prevent infection can help to reduce AMU in dairy production (Klaas and Zadoks, 2017).

2.2.3 Other pathogens

Prototheca spp. is an environmental alga, that causes mastitis and is associated to poor milking hygiene and contaminated water. It has been recognized in multiple countries and its incidence in dairy herds is increasing Worldwide. *Prototheca* spp. can spread from cow to cow like contagious pathogens if there are chronic infected animals in the herd (Jagielski et al., 2019). They are refractory to antibiotic treatment (Roberson, 2012).

Yeasts are organisms ubiquitous in the environment which are considered opportunistic pathogens of the mammary gland, causing mastitis when immunity is impaired. Sources of infection include the skin of the udder, udder secretion, milkers' hands, milking machines, treatment instruments, floor, straw, feed, dust, soil and sanitizing solutions. Mastitis caused by yeasts can become persistent and subsequently culling of these animals is the only option (Scaccabarozzi et al., 2011).

2.3. Mastitis prevention

In a context of societal pressure to reduce AMU and to maximize production in dairy farms, the adoption of preventive practices targeting the host and the environment to reduce the emergence of new IMI and the transmission of mastitis-causing bacteria (**MCB**) are becoming more common.

2.3.1 Host

A way to prevent mastitis is to select cows with characteristics that offer them more resistance to mastitis. Cows' teats are the first barrier against the entry of bacteria and selecting for characteristics such as the amount of keratin present (Capuco *et al.*, 1992), lower peak flow rate (Rupp and Boichard, 2003), or longer teat canal length (Hillerton and Lacy-Hulbert, 1995) reduce the risk of mastitis. Some of those characteristics, e.g. low peak flow rate, may not fit well in modern milking practices. Another way to prevent mastitis is to select sires with high breeding values for mastitis characteristics, such as udder conformation, SCC data and CM data. This means that the progeny of this animal will be more resistant to mastitis (Rupp and Boichard, 2003).

Another strategy to prevent mastitis is to increase cows' immunity. This can be done using an effective vaccination. Regarding *Staph. aureus* vaccine, several studies have been conducted and there is no commercially available vaccine for which there is convincing evidence of sufficient protection to justify its use (Rainard *et al.*, 2021). Some evidence showed that a commercial vaccine reduces the chances of transmission (mostly in first lactation animals) and improves the chances of cure (Schukken *et al.*, 2014), however other studies did not reach the same conclusion (Landin *et al.*, 2015). *E. coli* vaccine has also shown good results and is capable of reducing CM severity and milk yield losses. This vaccine even offers some protection against culling in cows with *Klebsiella* spp. mastitis (Bradley *et al.*, 2015). Recently, a *S. uberis* mastitis vaccine was launched, and this vaccine administered in the pre-calving period was able to reduce CM severity and milk yield losses in an experimental challenge. Further studies are still needed to assess its efficacy under field conditions (Collado *et al.*, 2018).

To adopt more specific strategies targeting the host, particularly for environmental mastitis, it is important to determine whether CM that occurs in early lactation is due to IMI that occurs during the dry period or during lactation. If IMI occurs in early lactation, nutrition may be inadequate. Good nutrition management can give cows more resistance to mastitis, allowing their immune system to combat better any IMI. It is important to have feed composition consistency, supply enough vitamin E and selenium, particularly when feedstuffs are grown in soils deficient in these elements or when animals are consuming stored forages (Smith *et al.*, 1997). Body condition score at calving is also important to avoid important negative energy balance and clinical or subclinical ketosis, which impairs udder defences (Suriyasathaporn *et al.*, 2000). If IMI has dry period origin, it is important to review DCT protocols and dry cow nutrition and hygiene. DCT was initially developed to allow long-term treatment of existing IMI without the need to discard milk (Neave *et al.*, 1969) and has subsequently been recommended as a tool to prevent new dry period infections in herds with low IMI prevalence (Schukken *et al.*, 1993).

However, this practice has high AMU and, although the relationship between AMU and the development of AMR in mastitis bacteria is complex and unclear, there is a potential selection for AMR. Therefore AMU in non-infected quarters is not necessarily a prudent use of antimicrobials (Scherpenzeel, 2017). In non-infected quarters usage of internal teat sealants (**ITS**) at dry-off prevents IMI during the dry period and has a protective effect on the incidence of new CM cases (Rabiee and Lean, 2013). In some studies, the use of ITS was as effective as DCT with antimicrobials in preventing new IMI (Huxley *et al.*, 2002). Is important to make it clear that if there are any existing IMI, ITS alone would not resolve them.

2.3.2 Environment

Environmental mastitis has become relatively more prevalent, whereas contagious mastitis has become relatively less important in dairy herds in the last decades due to the implementation of mastitis prevention programs. Cows' skin has *Staph. aureus* and NAS. Cows' faeces can contain *S. uberis* (Zadoks *et al.,* 2005), *E. coli* and *Klebsiella* spp. (Munoz *et al.*, 2006) and, occasionally, *Staph. agalactiae* (Jørgensen *et al.*, 2016; Cobo-Ángel *et al.*, 2018). Consequently, where there is a cow, there are faeces and MCB in the environment (Klaas and

Zadoks, 2017). Alleys, traffic lanes, water troughs, outdoor environments, and bedding, when contaminated with faeces are sources of MCB. Barns and milking parlours for lactating cows as well as heifer or dry-cow barns should be kept as clean and as "free" as possible from MCB. Improving hygiene, however, requires time and effort, and the economic margins in dairy production are often so narrow that such effort is compromised.

Measures such as flies control using pour-on synthetic pyrethroids directly on the udder and teats in high risk period and high risk areas, use of teat sealant and good pasture management are important to control and prevent Summer mastitis (Ahmad *et al.*, 2015; Blowey and Edmondson, 2010).

2.3.3 Milking parlour

In the milking parlour, the contagious transmission of MCB can be controlled through good milking parlour hygiene, early identification, and segregation of animals with IMI, treatment or culling of infected animals, and application of products or tools that reduce the probability of transmission, such as teat disinfectants.

Milking machine settings can account for up to 20% of new IMI (Mein, 2012). It is important to have a regular service of the milking machine, make sure that the vacuum is set correctly, avoiding liner slips, air suction and overmilking. Overmilking, which can be due to machine or operator error, can be responsible for teat-end hyperkeratosis which depletes cows' teat end defences and increases the incidence of mastitis (Pantoja *et al.*, 2020). Vacuum conditions may vary between clusters and liners, if not correctly adapted (Pyörälä, 2002) may be responsible for liners slips and air suction, which affects milking performance and teat condition (Besier and Bruckmaier, 2016).

Using teat-dips before and after milking reduces the incidence of IMI caused by environmental mastitis pathogens, and the incidence of IMI caused by opportunistic and contagious mastitis pathogens, respectively (Enger *et al.*, 2016). In Europe, the use of pre-dips containing disinfectants to reduce bacterial load prior to milking is rare or prohibited because of the risk for residues in milk. Regardless of whether a wet (pre-dip used) or dry (no pre-dip used)

pre-milking routine is adopted, it is important to monitor the efficacy of the procedure. If disinfectants are applied to dirty teats, they won't work (Munoz *et al.*, 2008). Milkers' hands, udder cloths and teat-dips can be sources of MCB (Klaas and Zadoks, 2017). *Serratia* spp. outbreaks have been associated with unhygienic handling of teat-dip (Muellner *et al.*, 2011) and unhygienic teat wipes have been a source of *Pasteurella* spp. (Klaas and Zadoks, 2017). Post-milking teat dips can reduce new IMI by 50%, however, it should be done every milking and must have a disinfectant with effective germicide action (Pankey *et al.*, 1984). Another indirect benefit of teat dips is that they can help to maintain and preserve a healthy teat skin. Nevertheless, in some parts of the world, in winter conditions post-dips should be carefully managed, as they can do more harm than good if there is a risk that they can cause frostbite. In this context, some powder-based teat dips or temporary discontinuation of teat dip use may be a preferred management option. However, there are no one-fits-all solution, as a change in dipping routine can result in a decrease in mastitis prevention against some type of pathogens but may result in an increase in others (Morrill *et al.*, 2019).

Automated milking systems are becoming more common worldwide; they are replacing traditional milking in the parlour due to labour shortages and have the potential to increase milk production by increasing milking frequency. Regarding mastitis control, automatic milking systems can have limitations with mastitis detection and teat cleaning procedures, which have been improved in recent years, through the improvement of equipment and operational settings. Automatic detection using sensors and machines also offer some advantages because they check every cow, every quarter and the milk produced. People often do not do that, and generally, machines are better than people at detecting changes in colour, milk temperature, and conductivity. Furthermore, these systems milk at quarter level avoiding overmilking or reducing the risk of cross-contamination among quarters of the same cow during the milking process and record a large amount of data that can be used to adopt strategic interventions (Hogenboom *et al.*, 2019). Shifting from conventional milking to automatic milking is generally associated with a deterioration of udder health performance, however careful interpretation of the origin of these problems is needed because the changes that occur are often broader than the milking system, and often include housing and laneway changes which can also affect udder health (Penry, 2018). Strategies to improve barn and udder hygiene, such as comfortable facilities, clipping the

udder hair, correct machine settings and cleaning, and milking intervals adapted to each animal's stage of production, can help to mitigate these challenges. Is important to not forget that despite being an automated system, the staff responsibilities are still significant (Hovinen and Pyörälä, 2011).

2.3.4 Barn

Used and unused bedding can be an important reservoir for environmental pathogens when contaminated with bovine faeces (Klaas and Zadoks, 2017). Different types of bedding are associated with different bacterial growth. Sawdust can be a source of *Klebsiella* spp. mastitis, but this type of mastitis can occur with any bedding material after faecal contamination has occurred (Ericsson Unnerstad *et al.*, 2009; Verbist *et al.*, 2011). Straw and peat can be a risk factor for *S. uberis* mastitis (Ericsson Unnerstad *et al.*, 2009). Composted bedded packs were associated with *Klebsiella* spp. outbreaks (Klaas and Zadoks, 2017). Recent research indicates that if this bedding is well managed by keeping the pack dry and the cows clean, and with the use of excellent teat preparation, it can be used with minimal effects on udder health (Leso *et al.*, 2020). Recycled manure solids are a rich source of nutrition for bacteria, even if they were free from coliforms before use. There is some concern that the use of recycled manure solids may contribute to AMR, and this may need further investigation (Leach *et al.*, 2015; Klaas and Zadoks, 2017). Inorganic bedding like sand can be a good option to reduce the environmental load of opportunistic pathogens, although faecal contamination will turn sand into a partially organic bedding material. Frequent cleaning of barn alley floor helps to improve cow hygiene (DeVries *et al.*, 2012), and proper alley scraping can make a difference between clean and dirty udders (Magnusson *et al.,* 2008).

If these preventive measures fail, an animal can develop IMI and CM, and to ensure animal welfare and reduce the economic impact of this disease, mastitis treatment options need to be considered along with the need to reduce AMU.

2.4. Mastitis treatment

Mastitis is the most common reason for AMU in dairy farms (Pol and Ruegg, 2007b), as it is the most common bacterial disease and farmers believe that antimicrobials are important for the success of the treatment (Swinkels *et al.*, 2015). However, AMU may lead to AMR, and high antimicrobial usage in livestock production has been critically questioned (WHO, 2015). Some countries, particularly in Northern Europe adopted regulations and restrictions on AMU several decades ago, e.g. a veterinarian must be consulted before starting a mastitis treatment (Wolff *et al.*, 2012). In other countries, e.g. The Netherlands, a reduction in AMU was enforced in recent years (Bos *et al.*, 2015). In the UK, antimicrobials are prescribed by veterinarians, but in mild and moderate CM cases farmers make their own treatment decisions. However, there are food standard schemes where the farmer is required to have an annual review of AMU with his or her veterinarian to provide guidance for responsible AMU (Red Tractor Assurance, 2018).

To preserve antimicrobial efficacy and safeguard human health, strategies that promote and ensure prudent use of antimicrobials in animal agriculture, including dairy production, are needed (Trevisi *et al.*, 2014; O'Neill, 2016). To reduce AMU, selective or targeted treatment can be used in lactating cows with CM (Lago *et al.*, 2011a,b; Vasquez *et al.*, 2017; McDougall *et al.*, 2018).

Treatment of CM during lactation differs between countries and farms. Ideally, it should be based on CM severity, and knowledge of causative pathogens and susceptibility profiles at cow or herd level. Mild and moderate CM treatment generally consists of the application of intramammary (**IMM**) antimicrobial ointment through the teat canal (Steeneveld *et al.*, 2011), possibly with concomitant administration of non-steroidal anti-inflammatory drug (**NSAID**) (McDougall *et al.*, 2009). IMM antimicrobial treatments are prescribed to eliminate the pathogen from the cow or at least from the quarter, whilst NSAIDs are administered to control inflammation and manage pain. Parenteral antimicrobial are generally recommended in severe CM (Suojala *et al.,* 2013), and should be administered to control bacteraemia and potential secondary infections.

Despite all the protocols and treatments available, there is no panacea regarding CM treatment. However, it is known that some cases are more likely to cure than others, and in some cases, probability of cure is so low that antimicrobial treatment may not be justified. Before developing this argument, is important to understand how CM cure can be defined and assessed.

2.4.1 Measures of cure

Treatment success and "cure" are often used interchangeably, although they do not mean the same. Cure does not necessarily need to be preceded by treatment as it can occur spontaneously. Clinical cure is based on CM detection and it occurs when milk and udder return to their normal aspect (Oliveira *et al.,* 2013). This type of cure is often used by farmers as the perceived treatment success, but it has limitations as CM can revert to SCM, detectable by elevated SCC or the presence of MCB in milk samples taken after treatment (Pinzón-Sánchez and Ruegg, 2011). Another measure of cure is the bacteriological cure (Degen *et al.*, 2015), which is associated with IMI detection and is defined as the absence of MCB in the milk sample taken 14 to 21 days after a previous milk sample where a MCB was detected, although different authors may use different test numbers or intervals for evaluation of bacteriological cure (Barkema *et al.,* 2006). Clinical cure and bacteriological cure are thus different outcome measurements, focussing on clinical signs and IMI, respectively. They can occur as a result of treatment or spontaneously and occur simultaneously or not. The bacteriological cure is probably harder to measure objectively, and not practical or economically viable to evaluate as an outcome measure of cure on-farm. However, bacteriological cure is often very important in research and used as the gold standard test. Another relevant measure in mastitis management is treatment success. In contrast to cure, this must be preceded by treatment. Examples of measures of treatment outcome are bacteriological and clinical cures, number of days milk is not saleable, recurrence of CM, retention of the animal within the herd, SCC, milk production (Pinzón-Sánchez and Ruegg, 2011).

Another important aspect to take into consideration is that dairy farming is an economic business. This has led to the concept of "economic cure", which is when the benefit of treatment is greater than its cost. This concept is rarely used explicitly but it is important for optimising

treatment decisions from the economic perspective of the business (Barkema *et al.,* 2006). Understanding these outcomes is important to make adequate management decisions and better understand the factors that influence treatment success.

2.4.2 Mastitis Management

Mastitis can be managed in multiple different ways which will depend on farmers' desired outcomes and may differ between countries and production systems. Selective CM treatment based on information from factors that affect treatment efficacy (host, herd, treatment and pathogen) will help to inform which CM are more likely to benefit from treatment. In other words, there is an opportunity to restrict AMU to CM cases where the spontaneous cure is significantly less than the expected treatment cure rate. Management of CM with protocols helps to simplify this task. In the following section, management recommendations to reduce AMU and increase treatment efficacy, without jeopardizing animal welfare and animal health, while trying to minimize costs will be presented based on literature. Management protocols should include the best evidence-based practice and adapted to each farm's characteristics. They should be periodically monitored, reassessed and modified as needed (Roberson, 2003).

After detecting a CM case, it is important to severity score each CM case, because severe and non-severe CM, which encompasses mild and moderate CM, are managed differently. If possible, information on the host and the pathogen should be considered to implement targeted or selective treatment (Roberson, 2012). When is not possible to adopt treatment decisions based on knowledge of the causative pathogen, treatment should be supported with available information from the cow (see section: **2.4.2.1** Host factors), and from the herd (see section: **2.4.2.2** Herd factors). This knowledge would help to choose the treatment approach adapted to cases that are most likely to benefit from treatment and reduce AMU by minimizing non-essential AMU. If antimicrobial treatment is not indicated, other cost-effective management options (as seen in previous section:**2.3.1** Host) should be considered to minimize unnecessary AMU and have the most economic benefit of the decision made.

2.4.2.1 Host factors

There are several factors at the host or quarter level that can affect the probability of a CM case to cure. This knowledge can be used in the decision-making process of CM treatment. After detecting CM in an individual animal, severity scoring must be used to differentiate CM cases in mild, moderate and severe (see section: 2.1 Types of mastitis), because severe cases are emergencies and should be treated immediately using the supportive treatment and parenteral antimicrobial (Roberson, 2012; Suojala *et al.,* 2013; Ruegg, 2017). By contrast, mild and moderate CM are not emergencies, and antimicrobial should not be administered until a cow's medical history and characteristics are reviewed, to identify animals that are likely to benefit from antimicrobial treatment. Questions and information regarding the cow need to be considered, such as "Is it a new case of CM or relapse?", "How many quarters are affected?", "What is the cow's stage of lactation, pregnancy status, and production value?", "Are there any other concomitant health problems?"

Increasing parity has been described in several studies to be associated with a low probability of cure. Studies on mastitis caused by *Staph. aureus* reported chances of cure in heifers and in older animals between 57-92% and between 27-67%, respectively (Pyörälä and Pyörälä, 1998; Taponen *et al.*, 2003a). Pinzón-Sánchez and Ruegg, (2011) reported a higher cure proportion in cows with low parities (≤ 3) (between 81-89.5%) than in cows with higher parities (60.7%). Adult cows, in third or greater lactation, are also 15.4 times more likely to have CM recurrence in any quarter than heifers (Pinzón-Sánchez and Ruegg, 2011). Parity affects the probability of cure from *S. uberis* CM in a similar manner (Samson *et al.*, 2016). A possible explanation for low cure rates in older animals includes a larger mammary gland size and larger volume of tissue to be cleared out, whilst a standard antimicrobial dose that is used for any udder size, and thus lower dose of antimicrobial per unit of udder volume in these animals (Barkema *et al.*, 2006). Another possible explanation, albeit without scientific evidence, is that older animals' immunity decreases with the age, a phenomenon known as immunosenescence, the gradual deterioration of the immune system brought on by natural age advancement (Samson *et al.*, 2016). Increased parity has also been described to be a risk factor for new dry period IMI, (14% for 2nd parity and 31% for ≥ 3rd parity animals) (Dingwell *et al.*, 2004). Cows from parity

4 or greater had 4.2 times higher risk to have a CM within 120 days in milk (**DIM**) of the following lactation than quarters from cows of parity 2 (Pantoja *et al.*, 2009).

Higher SCC before the development of CM is also associated with lower chances of cure (Bradley and Green, 2000; Pinzón-Sánchez and Ruegg, 2011), reported in specific studies on *Staph. aureus* (Owens *et al.*, 1988) and on *S. uberis* mastitis (Samson *et al.*, 2016). According to Bradley and Green (2009), the mean predicted probability of bacteriological cure after treatment when SCC before treatment was < 200.000 cells/ml is 40%; this value can decrease to 27 and 22%, if the SCC before treatment was between 200.000 to 800.000 cells/ml or above 800.000 cells/ml, respectively. History of high SCC is also associated with a probability of cure. Animals with 2 monthly milk recordings with SCC below 200.000 before treatment have a higher probability of cure than animals with multiple monthly milk recordings with high SCC for *Staph. aureus* (Barkema *et al.*, 2006) and *S. uberis* (Samson *et al.*, 2016). In situations with chronically elevated SCC in the previous lactation or more than four consecutive monthly milk recordings with high SCC in the current lactation, antimicrobial treatment may not be justifiable (Ruegg, 2017). The periods of increase in SCC may indicate that cows are chronically infected before the development of the CM, and generally, chronically infected cows have a lower response to treatment (Melchior *et al.,* 2006). Similarly, a longer duration of IMI for *Staph. aureus* as shown by consecutive culture-positive samples has been proven to influence cure rate (Sol *et al.*, 1994, 1997). This might be explained by fibrosis formation in udders with *S. uberis* IMI (Thomas *et al.*, 1994) and fibrosis and micro abscess formation in cases of *Staph. aureus* IMI (Erskine *et al.*, 2003).

Animals with a history of previous CM cases in the same lactation are less likely to experience bacteriological cure than those without prior CM (Pinzón-Sánchez and Ruegg, 2011; Samson *et al.*, 2016). In Pinzón-Sánchez and Ruegg, (2011) study the proportion of the first case of CM that resulted in cure were higher (86.5%) than the proportion of cases that were preceded by a previous CM case (51.9%). Therefore, animals that have a history of repeated treatments (more than 3) in the same lactation or animals that are concomitantly affected with other chronic diseases may not benefit from additional antimicrobial treatment (Ruegg, 2017). Another study demonstrated that the occurrence of CM at the previous lactation was a risk factor

for the development of CM at the beginning of the following lactation and also was associated with SCC status at the dry period (Pantoja *et al.,* 2009). That study followed several multiparous cows that experienced at least 1 case of CM in the previous lactation. It found that these animals were 4.2 times more likely to experience a CM case in the first 120 DIM of the following lactation than cows that completed the previous lactation without CM cases. This same study also reported that quarters that have high SCC at dry-off (SCC ≥ 200.000 cells/ml) were 2.7 times more likely to have a CM case in the first 120 DIM of the following lactation that quarters with low SCC at dry-off (SCC < 200.000 cells/ml) (Pantoja *et al.,* 2009).

Probability of cure from *Staph. aureus* IMI is reduced when multiple quarters are infected (Sol *et al.*, 1994; Osterås, Edge and Martin, 1999). Factors contributing to this may be the duration of infection, which is probably longer when multiple quarters are infected, the need to achieve cure in each quarter before the cow is cured, and the risk of within-cow transmission of the pathogen resulting in re-infection (Zadoks *et al.*, 2001). Mastitis in hind quarters is less likely to cure than front quarters, possible due to the volume of the gland, similar to the situation in older versus younger cows (Barkema, Schukken and Zadoks, 2006).

Several aspects will influence the economic benefit of a treatment, e.g. regulatory limits for bulk milk SCC, milk price, quality premium payments and penalties, labour costs, and quota systems for milk production or AMU. To optimise economic cure, such factors should be combined with cow history and cow characteristics that influence the probability of cure and information regarding animal's value. Milk yield (Pinzón-Sánchez and Ruegg, 2011a), reproduction status (Lehenbauer and Oltjen, 1998), milk quota situation of the farm, availability of replacement heifers (Steeneveld *et al.*, 2011) and retention pay-off value (Groenendaal, *et al.*, 2004) are important measures to inform decision making. Animals that are not expected to benefit from antimicrobial treatments based on their history or based on milk culture results and information of mastitis causing-pathogen (see **2.4.2.4** Pathogen factors section) can be managed with different options, which are culling, watchful waiting, dry-off of the affected quarter, early dry-off the cow, or segregation of the cow. Culling the cow is another approach, and the animal is removed from the herd because of sale, slaughter or salvage (Fetrow *et al.,* 2006). Watchful waiting involves hospitalization of the cow without antimicrobial treatment but NSAIDs can be

given to manage cow comfort. In this approach, milk is discarded until it returns to normal and animals' clinical progression is monitored. The watchful waiting should be applied for animals where the probability of spontaneous cure is high e.g., no history of previous CM, three consecutive milking records with SCC below 200.000, low parity, and non-severe CM (Ruegg, 2017).

In animals that have a single quarter with CM, with consecutive repetitive treatment failures, but still good milk yield in the other quarters, the affected quarter can be dried-off. With this approach, animals keep producing milk, normally 15% less (Steeneveld *et al.*, 2011). This might be a good approach when the economic and vital prognosis of the animal is not compromised, or for animals with high breeding value. With this approach, there is a decreased risk for the development of AMR and the animal can finish its lactation.

Early dry-off of the cow rather than the quarter is another option, particularly in a high-value pregnant cow with chronic SCM or CM. Early dry-off occurs when a pregnant animal is dried off more than 60 days before its due date. This approach is normally indicated for high value and high merit pregnant cows that have chronic CM or chronic SCM. Especially when segregation is not an option, which would reduce the risk of transmission within the herd, these animals can be dried-off earlier than usual. Segregation, i.e. separation of chronically infected animals from healthy animals is another option, particularly in farms where culling is not feasible due to economical constraints. These animals should be milked last, and milk should be discarded. They are candidates for future culling and should not be eligible for breeding (Ruegg, 2017).

For animals that are late in lactation and not pregnant, or lame, or that have other health problems or with low yield, culling may be considered (Royster and Wagner, 2015). In these animals, vital and/or economic prognostics are reduced and culling instead of treating CM may be a profitable option.

Culling is a decision that requires a balance in the herd to have economic animal health benefits. Poor culling management would allow the retention of older animals, lower producing cows, with poor udder health and higher rates of lameness and other potential chronic disease

and therefore retention of animals more likely to require extra AMU. However, if culling rates are too high this will shift the balance towards a greater number of younger, and healthier animals (De Vries, 2017). This can be positive in terms of reduction in AMU and managing udder health as an example of animal welfare. However, it raises societal concerns and ethical issues regarding environmental sustainability, climate change and animal welfare by decreasing animal longevity. Mature cows have higher milk yield, and heifers emit considerable amounts of greenhouse gases during the 2 first years of life when they are unproductive. Thus, decreasing culling rates by increasing the longevity of the herd increases environmental sustainability and is also associated with improved animal welfare and improved consumer perceptions of the dairy industry. This latter aspect is of extreme importance for the dairy sector. The market is regulated by consumer demands, and if there is a considerable shift away from the consumption of dairy products this may have ruinous consequences in the sector (Schuster *et al.*, 2020). In heifers, culling decisions are more difficult to make and treatment of CM is often recommended, because treatment in these animals has higher probability of cure, and the return on the investment in raising these animals is still to come (Barkema *et al.,* 2006).

2.4.2.2 Herd factors

The biology of CM in dairy cattle has not changed much over recent decades, although developments in genetics and at husbandry levels have led to significant changes in the predominant pathogen populations in developed countries (Barkema *et al.*, 2015). This has led to a decrease in the prevalence of contagious mastitis and a relative or absolute increase in the incidence of environmental mastitis (Klaas and Zadoks, 2017). In response udder health control programs need to focus more on the prevention of mastitis caused by opportunistic bacteria that reside in the environment often recognized as more complex than the control of mastitis caused by contagious pathogens. Herd data such as previous cases of CM, bulk tank SCC, individual test-day SCC, incidence, and prevalence of SCM, etc. continue to be a valuable parameter to measure, monitor and adopt strategies to manage and control mastitis at the herd level, based on simple epidemiological principles. They enable the identification of the predominant bacteria, to understand the most common modes of transmission and to categorize the herds as experiencing environmental or contagious patterns of disease, and to link them to a dry period

or lactating period origin. They allow to identify risk factors for transmission, offer an opportunity to reduce AMU, by allowing earlier review of the efficacy of treatment and control plans, and adoption of specific measures to prevent infection, or even to establish a prognosis before treatment decision. Usage of these records on the farm is still far from optimum, and in this section, some opportunities to improve record collection and assessment, as well some herd management changes to reduce AMU will be discussed.

Environmental mastitis problems often manifest as an increased number of cases of CM, rather than an increase in bulk milk SCC. CM data are of particular importance in herds where BTSCC are below 200.000 cells/ml, and if CM data is not recorded or available, just relying on bulk tank SCC may lead to the false impression that there are no udder health problems, as it is a good indication of SCM prevalence but not for CM because milk from animals with CM must be withheld from the tank.

CM records are often incomplete, as CM detection and recording can be difficult and subject to multiple barriers. Detection often requires milking personnel to modify their routines, it may slow down the milking process, or it may require employing an extra person in the parlour. Detection of an excessive number of CM in some circumstances can result in negative consequences for the milking staff, discouraging them from reporting CM cases that they have observed, and definitions may vary among farms and farm staff. Also, there can be confusion about the time between observations and when to consider a case to be a new case rather than the continuation of a previously observed CM case (Ruegg, 2003).

Information regarding the stage of lactation of each CM or SCM identifies high-risk period for infection and gives guidance in therapeutic decisions. Half of the enterobacterial CM cases that occur within the first 100 DIM arise in quarters that were already infected during the dry period (Bradley and Green, 2000). Thus, the association between dry-period IMI and lactational CM suggests that prevention of IMI during dry-period by reducing exposure to pathogens is important. The severity of CM is rarely recorded but such data can offer value in monitoring pathogen shifts. In a herd where most cases are caused by Gram-negative pathogens, a greater proportion of severe cases are expected. Mild cases have value in assessing the competency of milking staff to detect mild cases of CM. Other relevant aspect where CM and

SCC records can help to reduce AMU in the herd is in their usage to support treatment decision of SDCT (Scherpenzeel *et al.*, 2014).

Monitoring SCM is important because mastitis often fluctuates between SCM and CM. A cow with SCM is more likely to develop CM than uninfected cows (Pantoja *et al*., 2009). Information about lactation-average SCC and bulk tank SCC is used in mastitis control programs to improve milk quality related to SCM and is frequently available through milk collection. Individual test-day SCC patterns offer multiple advantages over bulk tank SCC. They can be used to monitor SCM in different cohorts (by lactation, DIM, age, and location of the dairy). Analysing monthly patterns of these selected cohorts can offer insight into areas needing investigation. Examples of such indices include the prevalence of SCM for first lactation and older cows; dynamics of intramammary infections across the dry period; the rate of new infections during lactation; and proportion of animals that are chronically infected. They are also very useful to monitor cure, assess CM, SCM and dry cow treatment (Rhoda and Pantoja, 2012), which can be used to identify and target specific management decisions to each animal such as segregation, culling, and treatment. Periodical milk recording offers the opportunity to capture long-term outcomes of mastitis treatment in a convenient and affordable way (Samson *et al.*, 2016). For CM and SCM treatment during lactation, monitoring SCC patterns is important to observe whether the current treatment protocols are adequate. This data can also be used to assess prognosis (see host factors), e.g. a review of SCC history that precedes a CM episode can inform the probability of cure for CM. It also can be used to inform treatment-decision, by providing some insight into the most likely aetiology that is causing the IMI and the CM episode. A CM case that is preceded by a long history of SCM is more likely to be caused by gram-positive bacteria, and normal milking records followed by a sudden case of CM is more likely to be caused by gram-negative bacteria (Ruegg and Pantoja, 2013).

Treatment of SCM may reduce the risk of cow-to-cow transmission, and the associated risk of new CM cases. The reduction in AMU for CM may offset the costs of treating SCM, depending on prevalence and transmission in a herd (Barlow *et al.*, 2013). Cost benefit analysis showed that the benefit of antimicrobial treatment in SCM caused by *Staph. aureus* was herd, cow and strain dependent. When contagious transmission of *Staph. aureus* is likely then

antimicrobial treatment of cows is often profitable if the right cows are selected based on risk factors for cure (see section:2.3.1 Host). When the contagious transmission of *Staph. aureus* is unlikely the treatment can be beneficial if it is of short duration and in animals that are more likely to cure, young and penicillin-sensitive animals (see section: 2.4.2.4 Pathogen factors). When the probability of transmission is low, the low intensity treatment regimens are profitable when appropriate cows are chosen (Swinkels *et al*., 2005).

2.4.2.3 Treatment factors

Antimicrobial treatment of non-severe mastitis is justified when the predicted probability of cure is higher with antimicrobial treatment than without (Ruegg, 2018). This is generally the case for gram-positive organisms such as staphylococci and streptococci but not for gram-negative organisms (Roberson, 2012; Suojala *et al.*, 2013; Lago and Godden, 2018). Because *E. coli* infections are generally limited to the superficial mucosal surfaces and have high spontaneous cure rates, antimicrobial treatment of mild and moderate cases is not needed (Suojala *et al.*, 2013). For streptococci and staphylococci, the probability of cure is significantly enhanced by antimicrobial treatment when compared to no treatment, although cow-factors and pathogen factors such as antimicrobial resistance also affect the probability of cure, as described respectively in sections **2.4.2.1** Host factors and **2.4.2.4** Pathogen factors. It is also common that some proportion of CM cases are culture negative, with variations between different studies and regions, but this can be responsible for 10 to 40% of CM (Roberson, 2003; Pinzón-Sánchez and Ruegg, 2011b; Oliveira and Ruegg, 2014). Other CM cases, can also be caused by organisms that are not susceptible to the antimicrobial approved, such as *Mycoplasma* spp., *Prototheca* spp. or yeast (Ruegg, 2018). Therefore, it has been estimated that in 50 to 80% of the CM cases, antimicrobial treatment might not be justifiable (Roberson, 2003), which supports that treatment decisions should be according to the mastitis-causing pathogen. And some studies, in North America and in Europe, evaluated the use of targeted treatment in CM, based on the differentiation between gram-positive pathogens and other causes of mastitis, showing that this approach has the potential to reduce AMU significantly without negative influence on udder health, production or culling (Lago *et al.*, 2011a,b; Mansion-de Vries *et al.*, 2014). Cephalosporins and fluoroquinolones are the only compounds with some evidence of beneficial

effects in the treatment of non-severe coliform mastitis (Schukken *et al.*, 2011a; Schukken *et al.*, 2013; Suojala *et al.*, 2013) but this evidence is not consistent (Pyörälä *et al.*, 1994; Ganda *et al.*, 2016; Fuenzalida and Ruegg, 2019). Moreover, 3rd and 4th generation cephalosporins and fluoroquinolones are HP-CIA and should not be used in animals (RUMA, 2020).

In severe cases, parenteral treatment is preferred and should be tailored to manage cardiovascular shock, reduce pain, inflammation and prevent bacteraemia. In these cases, priority should be given to correct dehydration, with hypertonic or isotonic intravenous fluids and oral fluids (Roberson, 2012). Parenteral antimicrobial are recommended to prevent bacteria spread in the bloodstream, and fluoroquinolones and 3rd and 4th generation cephalosporins are the recommended antimicrobial based on efficacy (Suojala *et al.*, 2013), although this contravenes WHO and RUMA recommendations.

In the past, the selection of the route of administration of antimicrobial was recommended according to the localization of the causative pathogen. For pathogens that remained in milk or ducts, such as *Strep. agalactiae*, *Strep. dysgalactiae* and NAS, IMM route was preferred. For pathogens that were recognised to penetrate the udder tissues, such as *Staph. aureus*, *Trueperella pyogenes* or *S. uberis* parenteral antimicrobial was recommended (Erskine *et al.*, 2003). However, in a randomized clinical control trial in mastitis caused by *S. uberis* parenteral treatment for 3 days achieved similar bacteriological cure rates to an aggressive IMM treatment (2 tubes per day) and higher than IMM treatment at labelled rates (1 tube per day) (80% vs 80% vs 64) (Hillerton and Kliem, 2002). This suggests that parenteral treatment is not better than IMM treatment. Parenteral antimicrobial and aggressive IMM used 14 times and 4 times more antimicrobial than the labelled dosage, and aggressive IMM use may be seen as the preferred balance between reducing AMU and achieving treatment efficacy (Hillerton and Kliem, 2002).

In a perspective of prudent AMU, parenteral antimicrobial should be limited to severe CM cases. IMM aggressive antimicrobial treatment should not be use, or only to cows that are carefully selected based on host factors. Likewise, extended treatments in mild and moderate CM should be limited to specific cases based on host or pathogen factors (Oliver *et al.*, 2003; Swinkels *et al.*, 2014) (see following sections). Intensive antimicrobial treatment regimens may

result in benefits, such as fewer follow-up treatments, less milk production loss and lower culling rates. These benefits sometimes do not outweigh the extra treatment costs (Steeneveld *et al.*, 2011), increased discarded milk, and increased risk of milk residues, with also potential selection for AMR (Barkema *et al.*, 2006). In addition to this, if not done properly, repeated infusions via the teat canal have higher risk for potential IMI (Gillespie *et al.*, 2002; Swinkels *et al*., 2005).

2.4.2.3.1 Gram-negative coliforms

Escherichia coli

The clinical signs are mainly a consequence of lipopolysaccharides (**LPS**), therefore, treatment should target LPS effects. In mild and moderate CM caused by *E. coli*, antimicrobial treatment is not needed, and administration of NSAIDs, frequent milking and fluid therapy should be the best approach. However, in severe CM cases there may be unlimited growth of bacteria or bacteraemia (Wenz *et al.*, 2001), and in these cases, antimicrobial treatment administered parenterally is recommended in order to control the infection and to increase cow survival. There are numerous antimicrobial molecules for systemic treatment, with variations in availability and its authorized usage among countries. These molecules include trimethoprim–sulphonamides, oxytetracycline, fluoroquinolones, cefquinome, and ceftiofur (Wenz *et al.*, 2001; Suojala *et al.*, 2013). Trimethoprim–sulphonamide combination at a high dose (48 mg/kg at 12-h intervals) has been recommended for treating coliform mastitis (Erskine *et al.*, 2003). However, concerns exist about the activity of these molecules in milk and the capacity to achieve therapeutic dosage following label dosage. Oxytetracycline efficacy in milk is also known to be decreased, due to strong chelate formation with casein, and dosage to achieve and maintain therapeutic concentrations are much higher than the label dosage; but no field studies were found in the literature to test this effect. A third-generation cephalosporin is authorized for systemic usage in both the USA and Europe. It diffuses poorly in milk, having zero milk withdrawal, and its usage for mastitis treatment in Europe is unauthorised. The key question here is whether parenteral treatment of cows with severe CM is interpreted as mastitis treatment (not permitted, not likely to work because there is no penetration of the mammary gland) or as sepsis treatment. A fourth-generation cephalosporin, cefquinome, is authorized for mastitis

treatment in some EU countries. A clinical trial that used this molecule systemically, alone or combined with IMM infusion, in comparison with a treatment group using IMM infusion with ampicillin-cloxacillin found that this cephalosporin improved clinical recovery and limited milk yield losses (Shpigel *et al.*, 1997). Systemic enrofloxacin resulted in faster bacteriological cure and significantly limited the milk loss and clinical cure of the affected quarter 24 hours post-treatment as compared with no treatment (Hoeben *et al.*, 2000), but enrofloxacin is a fluoroquinolone and hence a HP-CIA that should not be used in animals. Regarding treatment with ceftiofur administered in IMM infusions in mild and moderate CM caused by *E. coli* there is some evidence that this treatment increased bacteriological cure but had no benefits in milk production (Schukken *et al.*, 2011a). Thus, there is some evidence of the beneficial effects of treating *E. coli* CM with fluoroquinolones and cephalosporins, but this evidence is not consistent in other studies (Pyörälä *et al*., 1994; Ganda *et al.*, 2016; Fuenzalida and Ruegg, 2019). As it has seen previously, these antimicrobials are HP-CIA and their usage in livestock should be avoided. Non-steroidal anti-inflammatory drug treatment showed evidence of improved treatment efficacy in all *E. coli* CM, and their use in CM caused by these pathogens is recommended (Suojala *et al.*, 2013; McDougall *et al.*, 2016).

Klebsiella spp.

The reported spontaneous bacteriologic cure rates for this bacteria varied from 18 to 60% (Schukken *et al.*, 2011a; Ruegg, 2018; Fuenzalida and Ruegg, 2019a). In a recent study that evaluated the efficacy of IMM treatment in non-severe CM caused by gram-negative bacteria spontaneous bacteriologic cure rates of *Klebsiella pneumoniae* were lower than for *E. coli* CM (18 vs 97%). In this same study, non-severe CM caused by *Klebsiella pneumoniae* treated with IMM infusion of ceftiofur had higher bacteriological cure rates than CM cases caused by the same bacteria that did not receive any IMM treatment (78 vs 18%) (Fuenzalida and Ruegg, 2019a). Cases of non-severe CM caused by *Klebsiella* spp. and treated with a 5-day course of IMM ceftiofur were 8.5 times more likely to result in bacteriological cure than cases without treatment, with bacteriological cure similar to the previous study (57 vs 19%, respectively) (Schukken *et al.*, 2011a). Thus, non-severe CM caused by *Klebsiella pneumoniae*

may benefit from antimicrobial treatment in some situations and due to the poor prognosis, other management situations according to cow value may need to be considered.

2.4.2.3.2 Staphylococcus aureus

Spontaneous bacteriological cure for this pathogen can be as low as 0-11% (Ruegg, 2018), although estimated cure rates will depend on inclusion criteria. This pathogen is susceptible to multiple antimicrobials *in vitro*. However, *in vivo* cure rates can be variable. Cure rates can be very high, and treatment can be rewarding if the right cow is chosen and the infection is detected early. Treatment failure often occurs when the infection becomes chronic (Barkema *et al.*, 2006). Multiple reasons contribute to the poor response to treatment in chronic IMI caused by *Staph. aureus,* including its ability to survive inside neutrophils, the capacity to induce fibrosis and to form micro abscess (Erskine *et al.*, 2003), and the fact that it can adhere to the mammary gland epithelium and cause deep infection in the udder tissues (Schukken *et al*., 2011b). To improve treatment response, several studies with different antimicrobial classes, combinations of drugs, routes of administration and treatment duration have been conducted. However, comparisons between studies require caution, because definitions of cure, study designs, sample size, treatment length, molecules and formulations used, route of administration, animals' characteristics, etc. will vary among studies and often multiple different variables can be studied in a single study. When treating prepartum heifers cure rates were close to 100% for all the different molecules studied (penicillin-novobiocin, penicillin-streptomycin, cephapirin, tilmycosin, or a cephalonium-based products) (Owens *et al.*, 2001). For CM caused by penicillin-resistant isolates, both amoxicillin-clavulanic acid and spiramycin were equally ineffective with only 31% cure (Taponen *et al.*, 2003a). In another study of CM caused by *Staph. aureus*, the use of a 2nd generation cephalosporin resulted in a higher bacteriological cure rate than the use of cloxacillin (52.4 vs 12.5%) (Wraight, 2003). A study that investigated the same compound under different formulations of penicillin G, methicillin, and their esthers demonstrated that penethamate treatment (esther of penicillin G) for 4 days in SCM had higher cure rates (68.8 vs 56.5%) (Ziv and Storper, 1985). Drug combinations can have a synergistic effect, for example, penicillin and neomycin acted synergistically against *Staph. aureus* mastitis cases (Hensen *et al.*, 2000); or no different cure rates in CM using

penicillin G alone or combined with neomycin (Taponen *et al.*, 2003b). Some drug combinations are registered for use in mastitis, whereas other drug combinations may constitute extra-label usage, and require justification balancing benefit and risk.

Longer treatment is generally associated with higher probability of cure. For example in a small-scale study, SCM treated for 2-, 5- or 8-days had 13, 31, or 83% probability of cure, respectively (Gillespie *et al.*, 2002). Others reported 6, 56 and 86% cure for, no treatment, 2-day and 8-day treatment, respectively (Deluyker *et al.*, 2005). However, in another study of SCM, a 6-day extended treatment, with 3 cloxacillin tubes administered every 48h, had similar cure rates to no treatment (Shephard *et al.*, 2000). For CM, the evidence that extended treatment results in increased chances of cure is stronger than for SCM. In one study that compared a standard treatment of 3 IMM tubes administered every 12 hours with treatment that was extended for an additional 48 hours, the extended treatment was 2.3 times more likely to result in bacteriological cure than the standard treatment (Sol *et al.*, 2000). In another study of CM, 5-day treatment had a numerically higher chance of bacteriological cure than a 3 or4 day treatment (42 vs 29%) (Pyörälä and Pyörälä, 1998). The benefits of extended therapy protocols, such as higher proportions of cure, resulting in decreasing SCC, less risk of transmission (see section: 2.4.2.2 Herd factors), and improved saleability of milk, must be weighed against several disadvantages, among them are antimicrobial cost, discarded milk, increased risk for residues in the milk, and the potential increased risk of infecting the cow through repeated IMM infusions. Evidence regarding the route of antimicrobial treatment is not unanimous and sometimes difficult to interpret, as it includes different routes of administration, as well the presence of a second active molecule, or they often compare different molecules that are administered via different routes rather than the same molecule administered through a different route. Also, when comparing different routes of antimicrobial administration, the amounts of antimicrobial for parenteral use may be larger than for IMM, which has economical and potential public health implications regarding different cost-benefit and potential for the development of antimicrobial resistance (Barkema *et al.*, 2006).

2.4.2.3.3 Gram-positive catalase negative cocci

Streptococcus uberis

S. uberis treatment recommendations should be animal specific (see section: 2.4.2.1 Host factors) rather than pathogen specific because we have information on cow-level risk factors for cure (Samson *et al.*, 2016) but not on pathogen-level risk factors. Treatment should include antimicrobial as the probability of spontaneous bacteriological cure of these organisms is low with high recurrence if IMM antimicrobial infusions are not administered (Ruegg, 2018). When ceftiofur was administered for 2 days, in CM cases caused by *S. uberis* bacteriological cure rates were 43%. If treatment duration was extended to 5 or 8 days, bacteriological cure rates were 88% and 100%, respectively (Oliver *et al.*, 2004). Similar results were found for SCM caused by *S. uberis*, i.e. extended treatment was responsible for an increase in bacteriological cure (Oliver *et al.*, 2004). Pathogen factors, such as fibrosis and the ability to invade subepithelial and septal tissue, and lymphatic vessels and lymph nodes (Thomas *et al*., 1994), may explain why the response of *S. uberis* mastitis to treatment can be low, even after extended therapy (Milne *et al.*, 2005). As for *Staph. aureus*, the benefits of it must be considered relative to the increased costs of antimicrobial and discarded milk, and the increased risk of residues in milk and selection for antimicrobial resistance.

Streptococcus dysgalactiae

Strep. dysgalactiae, showed variable bacteriological cure rates that were lower than for *S. uberis* mastitis in CM studies from New Zealand (64.7-73.3% vs 87.7-89.8%) (McDougall, Agnew, *et al.*, 2007) and (73.7-100% vs 90.6-95.7%) (McDougall *et al.*, 2007), but higher in studies from SCM from United States (80 vs 67%) (Oliver *et al.*, 2004) and from Europe (7-26% vs 9-20%) (Deluyker *et al.*, 2005). Therefore these streptococci CM generally respond well to treatment but spontaneous bacteriological cure rates are generally poor (28-30%) (Ruegg, 2018). The differences between countries may be related to management differences, which is likely to be the case for *S. uberis* in New Zealand, or to strain differences, but little is known about *Strep. dysgalactiae* in this regard.

Streptococcus agalactiae

In the past, the prevalence of *Strep. agalactiae* IMI was reduced rapidly through "blitz" treatment. With this method, an entire herd or, more economically, all the infected cows in a herd are treated with antimicrobial. This was seen as an effective practice with cure rates from 70-90% (Erskine *et al.*, 2003). The success of this practice relied on bacteriological cure rates and the fact the bacteria was believed to be an obligate udder pathogen. However, as seen previously (see section: 2.1 Types of mastitis) this pathogen is found in environmental reservoirs and is also carried by a significant proportion of people (Skov Sørensen *et al.*, 2019). "Blitz" strategy has important costs, which are AMU, discarded milk and increased labour, as well as increased risk of milk residues, and the possibility IMI while infusing IMM treatment. Regarding the context of today where there are calls to reduce AMU because it potentially leads to AMR, this is not judicious or effective practice if not accompanied by other management changes to reduce prevalence and incidence of the infection e.g. good milk hygiene, good biosecurity and re-testing the treated, and if refractory to treatment, culling should be considered according to other host factors (Keefe, 2012).

2.4.2.3.4 Mycoplasma spp.

Treatment with antimicrobial is generally inadequate. *Mycoplasma* lacks a cell wall, meaning that they are inherently resistant to penicillin and cephalosporins due to the mode of

action of these molecules (Calcutt *et al.*, 2018). *In vitro* studies demonstrated that mycoplasmas were also resistant to all the main antimicrobial groups, including macrolides, tetracyclines, lincosamides, aminoglycosides, chloramphenicols and fluoroquinolones. Some of the resistance mechanisms are of the same nature as those described in cell-walled bacteria species, largely based on genetic point mutations (Lysnyansky and Ayling, 2016). As explained in the pathogen section (see section: 2.4.2.3.4 *Mycoplasma* spp.) these organisms have the capacity to disseminate in different body sites and to produce biofilms, meaning that treatment is rarely sufficiently thorough, or ineffective (Nicholas *et al.*, 2016). Therefore, antimicrobial treatment for mastitis caused by *Mycoplasma* is not justifiable economically or in terms of prudent AMU.

2.4.2.3.5 Minor pathogens

Bacteriological cure rates with penicillin for NAS range from 80-90%. However, for penicillin-resistant isolates it was shown that cure rates decrease by 20% compared to penicillin-sensitive isolates, which might indicate that spontaneous cure occurs commonly. Treatment duration was between 2 and 4 days and is recommended that 2-3 days of treatment can be used to treat NAS mastitis (Pyörälä and Taponen, 2009b). The authors of the cited review defend that antimicrobial treatment is not economically justifiable when a quarter has single isolation of NAS, particularly, if it also yields low numbers of bacteria in the milk sample, as these bacteria can have their origin in the teat skin and their detection may not constitute clear evidence of IMI. Spontaneous bacteriological cure for NAS has been reported to be as high as 70% (McDougall, 1998; Wilson *et al.*, 1999), and IMI by these bacteria have limited severity (see section 2.2.2.2 Non-aureus Staphyloccocci). It is recommended to limit antimicrobial usage for treating moderate-severe CM and quarters with persistent IMI (Pyörälä and Taponen, 2009b). In the same review, authors recommend that the antimicrobial chosen should be based on susceptibility testing accompanied by beta-lactamase testing, because resistance profiles of NAS can be quite variable, and some may even be methicillin-resistant (Sampimon *et al.*, 2011). In persistent infections, DCT at dry-off remains a good tool as cure rates at this stage are generally higher than in lactation (Pyörälä and Taponen, 2009b).

2.4.2.4 Pathogen factors

Apart from penicillin-resistance in *Staph. aureus*, relatively little is known about pathogen factors that affect treatment outcome. Existing knowledge is largely limited to AMR, capacity for adherence and invasion of the mammary gland tissues, and ability to form biofilms, as summarized in this section.

2.4.2.4.1 Antimicrobial resistance

Antimicrobial resistance of MCB can vary among countries and studies and often there is a lack of homogeneity of criteria to evaluate them, limiting the feasibility and the value of comparisons. For *Staph. aureus*, for example, resistance to β-lactam antimicrobials is well understood, and it is known that penicillin-resistant strains are far less likely to respond to any treatment than penicillin-sensitive strains (Barkema *et al.*, 2006). The mechanisms behind it, however, are not clear. A possible explanation is that penicillin-resistant genes are in pathogenicity islands that also encode virulence genes that affect the probability of cure. Another relevant aspect is that antimicrobials, specifically fluoroquinolones, can stimulate the dissemination of these genes. Thus, testing for β-lactamase production or penicillin sensitivity in *Staph. aureus* samples should be a prerequisite for treatment and done routinely (Taponen *et al.*, 2003a). Among these genes encoding β-lactamase, blaZ is the most common and is responsible for resistance to penicillin (Olsen *et al.*, 2006). Phenotypic testing for penicillin resistance is part of the diagnostic routine in some laboratories, and genotypic detection of blaZ is part of commercially available PCR-based mastitis diagnostics (Koskinen *et al.*, 2009).

For *E. coli*, a recent study demonstrated that differences in AMR can occur among different *E. coli* phylogenetic groups, although they may be uncommon or limited (Tomazi *et al.*, 2018). However, there are reports that some *E. coli* bacteria developed resistance to molecules that have been used for many years in dairies (ampicillin, streptomycin, sulphonamide, and oxytetracycline), but resistance to fluoroquinolones and cephalosporines is still uncommon (Suojala *et al.*, 2013). Other study using shows the opposite, that despite some antimicrobial have been in use for years for mastitis treatment, isolates from the most common

MCB, including *E. coli*, did not show increased resistance to these antimicrobials (Erskine *et al.*, 2002).

In a recent study in Canada of *Klebsiella* spp. isolates from CM milk samples just 42% of the isolates showed some AMR, with streptomycin and tetracyclines being the AB that showed some degree of resistance (Massé *et al.*, 2020)

2.4.2.4.2 Adherence/invasion

Host-adapted strains of *Staph. aureus* grouped in the clonal complex 97 are believed to be more unlikely to cure, possibly because they have a better capacity to survive in bovine mammary tissue (Rainard *et al.*, 2018). *In vitro*, *Staph. aureus* showed the capacity to evade neutrophils and to form capsules, able to adhere to mammary gland epithelial cells, capacity to evade macrophage killing and to resist to phagocytosis (Barkema *et al.*, 2006; Tassi *et al.*, 2015).

For *Strep. agalactiae* host-adapted clade, CC61 strain showed several virulence factors involved in adhesion, invasion of the pathogen in host cells and evasion of the immune system defences. Likewise, *Strep. dysgalactiae* and *S. uberis*, showed the ability to survive within mammary epithelial cell for extended periods without losing its viability *in vitro* (Calvinho *et al.*, 1998; Tamilselvam *et al.*, 2006). Adherence and invasion may protect bacteria from the effect of antimicrobials and the effect of the immune system, although some antimicrobials, e.g. penethamate iodide, can penetrate intracellularly (Almeida *et al.*, 2007). Intracellular invasion has also been proposed as the mechanism underpinning chronic *E. coli* IMI (Almeida *et al.*, 2011).

2.4.2.4.3 Biofilm

Biofilm formation has been demonstrated *in vitro* for some pathogens, including *S. uberis* and *Staph. aureus*, but results are highly dependent on the test system used. For example, *S. uberis* O140J was used as the negative control in one study and whereas it was considered to be a biofilm former in a different study (Crowley *et al.*, 2011; Varhimo *et al.*, 2011), *Klebsiella*

spp. isolates from bovine mastitis have been reported that up to 84% produced biofilm (Schönborn *et al.*, 2017), probably explaining its low and variable response to treatment.

For *Staph. aureus* the ability to form biofilm varied between different strains, with strains isolated from the mammary gland having better capacity to form biofilm *in vitro* than strains isolated from teat skin and milking unit liners, suggesting a potential risk factor for infection (Fox *et al.*, 2005). For both pathogens, the existence and the potential role of biofilm *in vivo* is a matter of debate (Fontaine and Smith, 2006).

2.5 Tests available in clinical mastitis

To implement targeted treatment in lactational mastitis it is important that a diagnostic test can differentiate mild-to-moderate gram-positive mastitis from other types of mastitis, that the test has the potential to be used as a point-of-care test, i.e. either on-farm as a cow side test or with turn-around time or service of 24 hours, which still can be achieved by some commercial diagnostic laboratories or veterinary practices (Malcata *et al.*, 2020). Milk samples must be collected aseptically to have meaningful culture results, otherwise, contaminants might indicate that treatment is needed and result in a poor reduction of AMU. Current diagnostic tests can detect mastitis, i.e. inflammation, based for example on biomarkers, and diagnostic tests that can detect the presence of a pathogen, based on bacteriological culture or molecular methods. The first can be an indirect measure of infection as most mastitis cases are due to IMI (Adkins and Middleton, 2018), while the latter is a direct measure for infection. Inflammation information without any pathogen-specific information and the presence of infection without evidence of inflammation may not be sufficient evidence to support treatment (Nyman et al., 2016).

Currently, diagnostics tests for on-farm use are based on bacterial culture, whereas culture-independent methods are available off-farm or under development for on-farm use.

2.5.1 Culture-dependent tests for pathogen identification

The conventional microbiological laboratory cultures have been the "gold standard" for mastitis testing (National Mastitis Council (NMC), 2017), though there is no consensus on how to interpret culture results (Dohoo *et al.*, 2011). This may lead to difficulties in interpreting studies where point-of-care tests have been evaluated using this test as the gold standard or reference test. Furthermore, routine culture may yield false bacterial identifications (Koskinen *et al.*, 2009), with an overall rate of erroneously identified bacteria between 7 and 37% across different laboratories (Pitkälä *et al.*, 2005). These numbers can increase if performed by persons with less diagnosis expertise. Another drawback of this system is the delay between the submission of milk samples and reporting the results (Lago *et al.*, 2011a,b). To address these limitations and societal calls to reduce AMU, many point-of-care tests or on-farm diagnostics have been developed. Multiple diagnostic tests with different characteristics for the classification of mastitis bacteria are commercially available. Performance of diagnostic assays can be evaluated based in scientific characteristics such as sensitivity, specificity and accuracy, and convenience aspects such as cost, ease of use and turn-around time.

There are currently on the market an array of distinct diagnostics (reviewed by Malcata *et al.*, 2020). They can be based on Petrifilm (McCarron *et al.*, 2009a; Mansion-de Vries *et al.*, 2014b), agar plates (Royster *et al.*, 2014a; Viora *et al.*, 2014), tube-test based systems (Leimbach and Krömker, 2018), or dip-slide plastic paddles (Malcata *et al.*, submitted). Some identify bacteria in broad diagnostic categories such as gram-positive and gram-negative e.g. VétoSlide (Malcata *et al.*, submitted), MastDecide (Leimbach and Krömker, 2018), Petrifilm (Mansion-de Vries *et al.*, 2014b), or the Minnesota easy culture-biplate (Royster *et al.*, 2014a). Other tests allow to identify bacteria to genus or species-level, e.g. VétoRapid (Viora *et al.*, 2014), or the Minnesota easy culture-triplate (Royster *et al.*, 2014a). Most novel tests include AB susceptibility testing (Jones *et al.*, 2019). Despite their distinct characteristics, all tests are more reliable when used to classify more broad categories, such as growth, gram-positive and gram-negative than when they identify bacteria at the genus or species level (Lago and Godden, 2018). Peer-reviewed studies reported sensitivities for detecting gram-positive bacteria that could range from 58.6% (Leimbach and Krömker, 2018) to 98% (McCarron *et al.*, 2009a), specificities from 48% (MacDonald, 2011) to 97% (Leimbach and Krömker, 2018), and

accuracies from 58.6% to 85.3% (Leimbach and Krömker, 2018). The inherent trade-off between sensitivity and specificity may limit the accuracy of the test, and pathogen prevalence in test-evaluations may influence the confidence intervals around the point estimate. Most early tests based on Petrifilm or agar were cheap but readings and outcomes were subject to users experience (McCarron *et al.*, 2009b). Recent market introductions, such as Mastatest (Jones *et al.*, 2019), include automatic reading to increase ease of use, in exchange for a higher cost. All current assays are based on bacterial growth, resulting in turn-around times of more than 16 hours. This may be enough to inform treatment decisions, but farmers would prefer a result with a turn-around time between less than 12 hours (Griffioen *et al.*, 2016). The use of on-farm testing raises concerns regarding health and safety or environmental impact. In some countries, these tests require a specialized laboratory and must not be used by laypeople (Kerwat, *et al.*, 2020). They are potential ways to propagate hazardous pathogens. Besides the biological hazard, these tests are often made of plastic and their usage can generate non-biodegradable waste.

The cost of the test does not seem to be a priority of dairy farmers (Griffioen *et al.*, 2016), despite very low-profit margins that often affects the sector. There is some controversy regarding the economic benefit of on-farm testing, which may be herd and pathogen dependent (Cha *et al.*, 2013; Down *et al.*, 2017), and also regarding animal welfare impacts resulting from postponing treatment until having the results (Down *et al.*, 2017). Despite that, on-farm testing is recognized as a suitable opportunity to support targeted treatment of CM and reduce AMU. A recent study also showed that delaying treatment had no detriment to animal health (Bates *et al.*, 2020).

Most tests were designed to identify pathogens to genus or species level, but farmers may be more interested in advice on which antimicrobial to use (Griffioen *et al.*, 2016), and information at gram-level might be enough to implement targeted treatment decisions according to farmers expectations. Data on uptake of on-farm testing are scarce, but in a study from the Netherlands performed a few years ago, only 2% of farmers based treatment decisions on culture results (Griffioen *et al.*, 2016).

2.5.2. Culture-independent tests for pathogen identification

Culture-independent methods as the name indicate do not need culture and could have a faster turn-around time. They are not available on-farm yet, and currently cost more than culture.

2.5.2.1 DNA based methods

Polymerase chain reaction (PCR) can be used for the identification of MCB directly from milk. The PathoProof mastitis PCR assay was the first commercially available DNA-based method for pathogen detection directly from milk, and additional assays have become available since then. The initial version was a multiplex real-time test kit that can detect up to 11 different bacteria in the milk along with the β-lactamase gene (the blaZ gene) responsible for staphylococcal penicillin resistance (Koskinen *et al.*, 2009). It is important to associate detection of this gene with another test as the presence of the gene alone is not sufficient to determine whether is associated with *Staph. aureus* or NAS (Koskinen *et al.*, 2009). These real-time tests do not require a culture step and the total analysis can be done within 3-4 hours, which can be used to support targeted treatment CM. The use of PCR allows the detection of growth-inhibited, dead bacteria, which is often presented as an improvement in sensitivity compared to culture. However, whether the detection of DNA bacteria in culture negative samples requires antimicrobial treatment is questionable (Nyman *et al.*, 2016).

Another DNA-based test that has been evaluated for direct detection of mastitis pathogens in milk is loop-mediated isothermal amplification (**LAMP**). This test uses different chemistry than Polymerase chain reaction (PCR) and requires less sample preparation. It is less sensitive to inhibitory substances present in biological samples and may be applicable under field conditions (Bosward *et al.*, 2016). In research studies, this test can give fast, cost-effective pathogen identification. It can be converted into a pregnancy test-like a lateral flow device, which could be a rapid on-farm diagnostics option (Cornelissen *et al.*, 2016). A major challenge for on-farm molecular diagnostics is the existence of many pathogens species and AMR genes that may be present in milk or in mastitis pathogens, and it is difficult to design tests that incorporate primers or probes for all potentially relevant targets.

2.5.2.2 Diagnostic tests to detect inflammation

Current tests to detect inflammation for on-farm use include California mastitis tests, other measurements of SCC, measurement of the electrical conductivity, or the lactate dehydrogenase activity. Acute-phase proteins may also serve as mastitis biomarkers, such as haptoglobin (Åkerstedt *et al.*, 2009), serum amyloid A (Eckersall *et al.*, 2006), N-acetyl-β-D-glucosaminidase (Pyörälä *et al.*, 2011), lipopolysaccharide-binding protein (Schroedl *et al.*, 2001) and cathelicidins (Addis *et al.*, 2016, 2017). SCC and electrical conductivity are widely used on-farm whereas the use of acute-phase proteins is limited to veterinary or research laboratories. Markers of inflammation have limited value to support targeted treatment of CM because they lack pathogen-specificity. If biomarkers indicative of causative agents could be identified, this could form the basis of a test that is more rapid than culture and with the ability to differentiate between the need for treatment (mild-to-moderate CM due to gram-positive organisms) and no treatment (other mild-to-moderate CM).

2.6. Aims and objectives

The aim of the original research described in this thesis is to investigate the scientific characteristics (sensitivity and specificity) of different tools to support targeted treatment of bovine CM, considering a culture-based and a culture-independent approach.

Objectives

1. Culture-dependent assay

To evaluate the performance of a simplified test against a reference test consisting of bacteriological culture and determination of species identity using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF MS)

To compare the performance of the simplified test against a commercially available comparator test that is commonly in use in author's practice.

A. By assessing the difference among both tests
B. By assessing agreement between tests

2. Culture-independent assay

To develop a reproducible and consistent western blot protocol for a potentially pathogen-associated biomarker of mastitis, cathelicidin.

To assess if cathelicidin can be used to differentiate mild-to-moderate gram-positive CM from other forms of CM.

3. Because uptake of tests is determined by scientific characteristics as well as convenience aspects of tests and farmer behaviour, the general discussion will describe those aspects of the uptake of targeted treatment approaches and the barriers and drivers for uptake of on-farm diagnostics.

Chapter 3. Laboratory based evaluation of a simplified culture system as tool for targeted mastitis treatment

3.1. Introduction

Presence of mastitis in dairy herds causes production inefficiency (Halasa *et al.*, 2007) and animal welfare problems (Byrd *et al.*, 2017). Its control is the most frequent reason for AMU in lactating and non-lactating dairy cows (Pol and Ruegg, 2007b) with several consequences, including discarded milk (Ruegg, 2003), presence of antimicrobial residues in milk (Garcia *et al.*, 2019) and potential for the development of AMR (Pol and Ruegg, 2007a; Oliver and Murinda, 2012). High AMU in livestock production has been critically questioned (WHO, 2015). In order to preserve antimicrobial efficacy and safeguard human health, strategies that promote and ensure prudent use of antimicrobials in animal agriculture, including dairy production, are needed (Trevisi *et al.*, 2014; O'Neill, 2016).

To reduce antimicrobial use, selective or targeted treatment can be used, both in dry cows (Vanhoudt *et al.*, 2018; Vasquez *et al.*, 2018; Lipkens, 2019) and in lactating cows with CM (Lago *et al.*, 2011a,b; Vasquez *et al.*, 2017; McDougall *et al.*, 2018). Antimicrobial treatment of non-severe mastitis is justified when the predicted probability of cure is higher with antimicrobial treatment than without antimicrobial treatment (Ruegg, 2018). This is generally the case for gram-positive organisms such as staphylococci and streptococci but not for gram-negative organisms (Roberson, 2012; Suojala *et al.*, 2013; Lago and Godden, 2018). For example, *E. coli* infections are generally limited to superficial mucosal surfaces with more than 75% spontaneous cure rates (Suojala *et al.*, 2013). Other CM, caused by *Mycoplasma* spp., *Prototheca* spp. or yeast are intrinsically resistant to treatment (Ruegg, 2018).

The use of selective treatment for CM in lactating cows (please see section **2.4.2.3** Treatment factors), based on the differentiation between gram-positive pathogens and other causes of mastitis, has the potential to reduce AMU significantly without negative influence on udder health, production or culling (Lago *et al.*, 2011a,b; Mansion-de Vries *et al.*, 2014).

Cephalosporins and fluoroquinolones are the only compounds with some evidence of beneficial effects in treatment of non-severe coliform mastitis (Schukken *et al.*, 2011a; Schukken *et al.*, 2013; Suojala *et al.*, 2013) but this evidence is not consistent (Pyörälä *et al.*, 1994; Ganda *et al.*, 2016; Fuenzalida and Ruegg, 2019). Moreover, the World Health Organization classified both of these antimicrobial classes as HP-CIA for human medicine (WHO, 2019) and their use in veterinary medicine is discouraged. Thus, while there may be tension between the imperative to protect animal welfare and the need to safeguard public health, the evidence base for the value of antimicrobial treatment of mild to moderate gram-negative CM is weak and societal concern about such use is getting stronger. This has led to the development of an array of diagnostic assays for on-farm classification of mastitis pathogens to support selective treatment.

Numerous culture-based detection kits for the classification of mastitis pathogens have been described and commercialized. The performance of diagnostic assays can be evaluated using a range of criteria, such as the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) (Kosack *et al.*, 2017), which cover both scientific characteristics such as sensitivity, specificity and accuracy, and convenience aspects such as cost, ease of use and turn-around time. For test characteristics please see section: **2.5.1** Culture-dependent tests for pathogen identification.

Data on the uptake of on-farm testing are scarce. In a recent study from The Netherlands, only 2% of farmers based treatment decisions on culture results (Griffioen *et al.*, 2016). Availability of an affordable, user-friendly and simplified test that can differentiate gram-positive organisms from other causes of mastitis will potentially improve uptake of on-farm testing. The need for reliable and simple testing to differentiate between gram-negative, gram-positive or culture-negative samples was also recognized for point-of-care diagnosis of bacteriuria in pregnant women, leading to the development of the Uricult dip-slide (Van Dorsten and Bannister, 1986). The dip-slide is a plastic paddle with two selective media that can be dipped in a liquid sample such as urine or milk and allows for subsequent growth of either gram-positive or gram-negative organisms. The dip-slides can be used in a doctor's office (Van Dorsten and Bannister, 1986) or veterinary practice (Zadoks, personal communication) but I am not aware of any formal evaluation of the performance of such assays for the diagnosis of mastitis.

The aim of the current study was to evaluate the laboratory performance of a simplified slide test for bovine mastitis against a reference test consisting of bacteriological culture and determination of species identity using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Because assay performance may be influenced by the population under study, particularly the proportion of gram-positive and gram-negative organisms in the CM case selected for test evaluation, a commercially available plate-based test previously evaluated (Viora *et al.*, 2014) was used to benchmark the new simplified slide test.

3.2. Material and Methods

3.2.1 Regulatory compliance

This research was approved by the Ethics and Welfare Committee of the School of Veterinary Medicine of The University of Glasgow, UK (Ref 50a/16).

3.2.2 Sample size calculation

Sample size for evaluation of Sensitivity and/or Specificity of a diagnostic test was calculated using the formula: $n = \frac{\left(z_{\frac{\alpha}{2}}\sqrt{p_0(1-p_0)}+z_{\beta}\sqrt{p_1(1-p_1)}\right)^2}{(p_1-p_0)^2}$

According to this formula, n = sample size, P0 = denote the pre-determined value of sensitivity or specificity of new diagnostic test, P1 = is the value of sensitivity (or specificity) under alternative hypothesis. With (1 - α) % confidence level and (1 - β) % power for detection an effect of P1 - P0 using normal approximation as a general rule. $Z\frac{\alpha}{2}$ and $Z\beta$ denote the upper $\frac{\alpha}{2}$ and β percentiles of standard normal distribution and α and β are the probabilities of type I and type II errors respectively with confidence level (Z = 1.96 for 95% CI) (Hajian-Tilaki, 2014). Assuming a test sensitivity of 90% compared to the value of 100% for the reference test and a power of 80% the required sample size was 73.

Sample size for estimation of prevalence of gram-positive bacteria was calculated using the formula n=(Z^2 P(1-P))/d^2 , where n = sample size, Z = value from a standard normal distribution corresponding to a desired confidence level (Z = 1.96 for 95% CI), P = expected prevalence or proportion and d = desired precision (Daniel and Cross, 2013) as implemented in Excel (Naing, Winn and Rusli, 2016). Based on the range of prevalence's reported for gram-positive organisms in mastitis cases in different studies, e.g. 34% (Viora *et al.*, 2014), 44% (Lago *et al*., 2011a,b) and 49% (Milne *et al.*, 2003), a prevalence of 50% was assumed as it is the "worst case" scenario for sample size estimation (Naing, Winn and Rusli, 2016). For this prevalence and a precision of 0.2, the required sample size was 133.

3.2.3 Sample collection

Seven dairy farms in Scotland were selected for participation based on herd size, location and willingness to cooperate in the study (Table 3-1). Farm staff, including milkers and herd managers, were recruited to participate in the study and trained to identify CM cases and to classify them as mild (presence of abnormal milk such as milk with clots or flakes or serous milk), moderate (presence of signs of udder tissue inflammation: hardness, swelling, redness, heat or pain) or severe (animals with additional systemic signs of disease, such as fever, tachycardia, tachypnoea, dehydration, anorexia and decreased ruminal function) (Pinzón-Sánchez *et al.*, 2011a). They were also taught how to collect milk samples aseptically according to National Mastitis Council recommendations (NMC, 2017). The trained staff cleaned, pre-dipped and dried the teat, fore-stripped 2 to 3 times, scrubbed the teat using swabs and surgical spirit and collected milk in 30 ml sterile universal containers (Henry Schein, Melville, United States of America) held in a diagonal position and without touching the cap to avoid contamination. Universal containers are the most used milk sampling vessels in my practice area. No preservative was added to the milk samples.

Quarters with macroscopic changes in milk were sampled regardless of mastitis severity. If multiple quarters of a cow were affected simultaneously, all affected quarters were sampled. If a second episode of CM occurred in the same cow, she could be included again, regardless of whether the second episode occurred in the same or a different quarter. Any CM episode in the

same quarter occurring >14 days after the previous episode was considered a new CM case, regardless of the pathogen isolated. If the second CM episode occurred in the same quarter within 14 days with a different etiologic agent than that of the first episode, it was also considered a different CM case (Hertl *et al.*, 2014). Animals were eligible for inclusion in the first week after calving but no animals included were within 14 days of administration of antimicrobial products.

Samples were collected from January to May 2018. They were stored frozen on-farm (-20°C), and once a week, they were transported by me to Glasgow University's Veterinary Diagnostic Services laboratory where they were stored frozen (-20°C) until processing. All samples were cultured within 4 weeks from CM detection.

Table 3-1 Farms participating in sample collection to evaluate potential on-farm diagnostics for clinical mastitis.

Farm	Lactating cows (n)	Samples collected (n)	Bulk tank SCC (×1000 cells/mL)	Clinical mastitis incidence per 100 cows per year	Milking Frequency (/day)	Milking parlor type	Bedding
1	795	58	140	28	3	52-point rotary	sand
2	549	30	180	30	3	24 x 48 SwingOver herringbone	oat husks and lime
3	496	33	230	60	3	48 x 48 herringbone	sawdust and lime
4	607	5	145	40	3	24 x 48 SwingOver herringbone	recycled manure solids
5	304	8	195	45	2	24 x 24 herringbone	oat husks and lime
6	296	12	280	65	2	24 x 48 SwingOver herringbone	sawdust and lime
7	276	10	190	45	2	44-point rotary	sawdust and lime

3.2.4 Reference test

Samples were thawed at ambient temperature for up to 8 hours and processed simultaneously using the reference test, the simplified slide test, and the commercially available plate-based comparator test as described in the following two sections. I was blinded to the reference test result. For consistency, all media were inoculated and read by me. Bacteriological culture (NMC, 2017) with subsequent determination of species identity using MALDI-ToF MS was used as the reference test. Sheep blood agar (5% vol/vol; SBA) and MacConkey agar number 3 plates (E&O Laboratories Limited, Bonnybridge, Scotland) were inoculated with 0.01 ml of milk each using disposable sterile calibrated plastic loops. Plates were incubated at 37°C in aerobic conditions and examined after approximately 48 hours. IMI definition was of 100

colony-forming unit per mL of milk and was counted for each morphotype. Samples that did not yield growth of visible colonies were considered negative for mastitis-associated pathogens. Samples that yielded three or more colony types were considered contaminated and excluded from data analysis in accordance with NMC guidelines. For the remaining plates, each morphotype was identified by standard laboratory methods (NMC, 2017) including colony morphology, growth on MacConkey, catalase test, and haemolysis. In these plates, each colony type was sub-cultured onto half of an SBA plate for purification. From each pure culture, a colony was selected and grown in 2 ml of Brain Heart Infusion broth for 24 hours at 37°C in aerobic conditions without shaking. The isolates were preserved with 15% glycerol (v/v) in cryovials at -80°C and submitted to an external laboratory (Laboratoire de Microbiologie, Vétoquinol SA, Lure, France) for species identification by MALDI-ToF MS analysis, using Vitek-MS and V3.1.0 database (bioMérieux, Marcy-l'Étoile, France).

3.2.5 Slide test

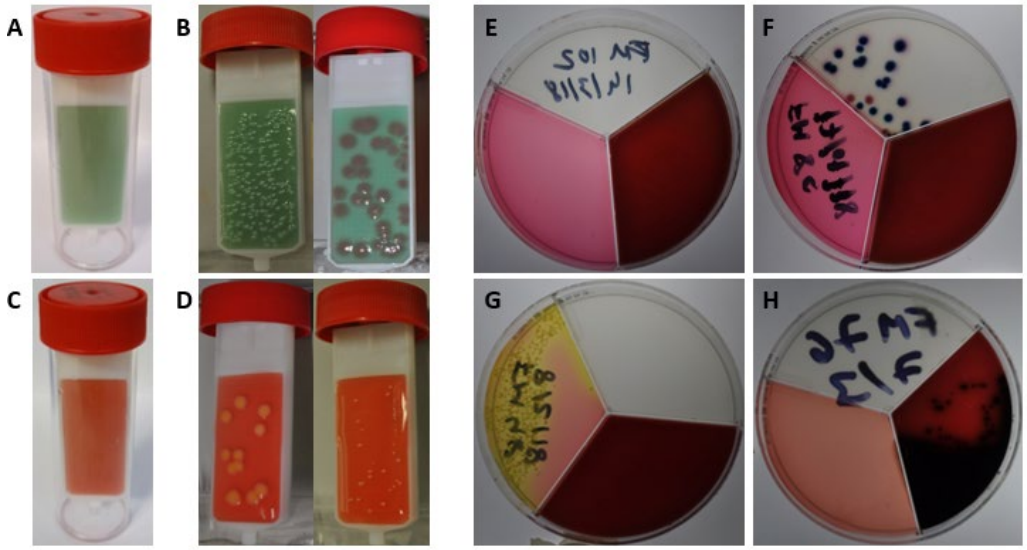
The slide test consists of a double-sided plastic slide with two selective media, i.e. a green side selective for gram-negative bacteria and a red side selective for gram-positive bacteria (**Figure 3-1**).

Media of the simplified slide test (VétoSlide, Vétoquinol, Lure, France) were inoculated by applying milk directly to each side using cotton wool swabs (approximate volume 0.1 mL) (Lago and Godden, 2018) to moisten the entire surface of the media, as per manufacturer's instructions. The inoculated slides were incubated at 37°C in aerobic conditions and examined after approximately 48 hours. When at least one colony was visible, the sample was considered positive (Dohoo *et al.*, 2011). Based on the manufacturer's guidelines, any growth on the green media was considered to indicate the presence of gram-negative bacteria and red colonies on the green media were considered to be *E. coli*. Growth on the red media was considered to indicate the presence of gram-positive bacteria (**Figure 3-1** A-B). When there was growth on both media, it was considered to indicate mixed infection with gram-positive and gram-negative bacteria. Guidelines for interpretation of results as indicative of contamination were not given so samples were never classed as contaminated based on the slide test.

3.2.6 Comparator test

Plates for the comparator test (VétoRapid, Vétoquinol, Lure, France) were chosen for benchmarking because it is the most commonly used on-farm test in my dairy community and was previously evaluated in similar study settings (Viora *et al.*, 2014). These plates contain three sectors of selective indicator media, i.e. for gram-negative organisms (sector 1), staphylococci (sector 2) and streptococci and enterococci (sector 3) (Viora *et al.*, 2014) (**Figure 3-1** E-H). Plates were inoculated with 0.01 ml of milk per sector using disposable sterile calibrated plastic loops. The inoculated plates were incubated at 37°C in aerobic conditions and examined after approximately 48 hours. Based on the manufacturer's guidelines, it was possible to identify eight of the most common mastitis-associated pathogen species or genera: *E. coli* (dark blue colonies on sector 1), *Klebsiella* spp. (red-purple colonies on sector 1), *Staph. aureus* (yellow or golden colonies and yellow or golden discolouration of agar on sector 2), non-aureus staphylococci (NAS; clear colonies and no change in the agar colour on sector 2), *S. uberis* (black colonies and blackened agar on sector 3), *Enterococcus* spp. (black colonies and agar on sector 3 together with pinhead yellow colonies and discolouration of sector 2), *Strep. dysgalactiae (*clear colonies on sector 3 with green coloured corona at 48 hours and red-brown agar colouration) or *Strep. agalactiae* (clear colonies on sector 3 with clear-bright corona at 48 hours and red-brown agar colouration). Samples that did not yield visible colonies on the comparator test were considered negative for mastitis-associated pathogens. As for the slide test, a contaminated category was not specified by the manufacturer.

Figure 3-1 Test kits. On the left: Slide test. The green media (A) allow for detection of gram-negative bacteria, with *Escherichia coli* growing as red colonies and other species as white colonies (A, B). The red media allows for detection of gram-positive bacteria. (D) Bacterial growth on red media. E. Commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) with 3 selective indicator media. (F) Growth in the sector selective for gram-negative bacteria. (G) Growth in the sector selective for staphylococci. (H) Growth in the sector selective for streptococci and enterococci.



3.2.7 Data analysis

Samples that were contaminated based on the reference test, or that contained isolates that were non-identifiable by MALDI-ToF MS, were excluded from the evaluation of diagnostic test performance. All other culture-positive and culture-negative samples (n = 130) were included in the calculation of sensitivity, specificity, accuracy and predictive values for growth, gram-positives, gram-negatives and *E. coli*. The reference test was used to classify results from the slide test and comparator test as correct or incorrect. For culture-positive samples with gram-positive or gram-negative species as identified by the reference test, matching results from the slide test or the comparator test were considered true positives (TP) and non-matching results were considered false negatives (FN). For example, a sample yielding *Staphylococcus haemolyticus* with the reference test, gram-positive growth on the slide test, and NAS on the comparator test was considered a true positive (TP) for growth of gram-positive organisms in the two on-farm tests, a true negative (TN) for gram-negatives or *E. coli*. A sample that yielded

no growth in the reference test, gram-positive results in the slide test and *Staph. aureus* in the comparator test was interpreted as false positive (FP) at growth and gram-positive levels in both tests and as true negative (TN) at gram-negative and *E. coli* levels. Additional examples of interpretation of tests results as TP, TN, FP or false negative (FN) are given in **Table 3-2**. From those classifications, sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows: Se = TP/(TP+FN), Sp = TN/(FP+TN), Ac = (TP+TN)/n, PPV = TP/(TP+FP), NPV = TN/(FN+TN).

To evaluate the potential of the test kits as treatment decision support tools, the calculations were repeated using a subset of samples, originated from non-severe CM cases only (n = 109), that were neither contaminated nor contained an organism that could not be identified by MALDI-ToF MS and the outcome was expressed as "treatment". This outcome is equivalent to gram-positive growth (one or two colony types) against not gram-positive. The latter category includes gram-negative bacteria, non-bacterial growth, and culture-negative results.

Statistical analysis was performed in Excel (Microsoft Corp., Redmond, USA) using tabular methods done by myself, and in R with the guidance of a statistician (R Core Team, 2019). Epidemiological parameters were expressed as percentages with 95% Wilson type confidence intervals (**CI**), calculated using the Hmisc package in R (Harrel Jr and Dupont, 2019). Wilson intervals are preferred over exact intervals and Wald (normal approximation) type intervals, as they have coverage probability closer to the nominal value (Agresti and Coull, 1998) and confidence limits that do not exceed the boundaries of the unit interval. The parameter estimates for the slide test and the comparator test are not independent because they are derived from the same sample. To account for this dependence when considering differences between estimates of Se, Sp, Ac, PPV and NPV for the two tests, Wald type confidence intervals were calculated using formulae derived from Kosinski (2013). If the 95% confidence interval for the difference between tests excluded zero, test performance was considered significantly different.

Agreement between tests was expressed as Cohen's kappa coefficient (κ) and considered almost perfect ($\kappa > 0.8$), substantial ($0.61 \leq \kappa \leq 0.8$), moderate ($0.41 \leq \kappa \leq 0.6$), fair ($0.2 \leq \kappa \leq 0.4$), or slight ($\kappa < 0.2$) (Dohoo *et al.*, 2009).

Table 3-2 Examples of possible results in the reference test and the simplified slide test under evaluation (VétoSlide, Vétoquinol, Lure, France) with respective classification as True positive (TP), True Negative (TN), False Positive (FP) and False Negative (FN).

Examples of possible results in the reference test	Examples of possible results in the VétoSlide	Growth	Gram-positive	Gram-negative	*Escherichia coli*	Treatment
1 gram-positive	gram-positive	TP	TP	TN	TN	TP
1 gram-positive	gram-positive and gram-negative[1]	TP	TP	FP	TN, FP[1]	TP
1 gram-positive	gram-negative[1]	TP	FN	FP	TN, FP[1]	FN
1 gram-positive	No growth	FN	FN	TN	TN	FN
2 gram-positives	gram-negative[1]	TP	FN	FP	TN, FP[1]	FN
2 gram-positives	No growth	FN	FN	TN	TN	FN
1 gram-positive and 1 gram-negative[1]	gram-positive	TP	TP	FN	TN, FN[1]	TP
1 gram-positive and 1 gram-negative[1]	gram-positive and gram-negative[2]	TP	TP	TP	TN, FN[1], TP[1,2], FP[2]	TP
1 gram-positive and 1 gram-negative[1]	gram-negative[2]	TP	FN	TP	TN, FN[1], TP[1,2], FP[2]	FN
1 gram-positive and 1 gram-negative[1]	No growth	FN	FN	FN	TN, FN[1]	FN
No growth	gram-positive	FP	FP	TN	TN	FP
No growth	gram-positive and gram negative[1]	FP	FP	FP	TN, FP[1]	FP
No growth	No growth	TN	TN	TN	TN	TN

[1,2]If *E. coli* detected.

3.3. Results

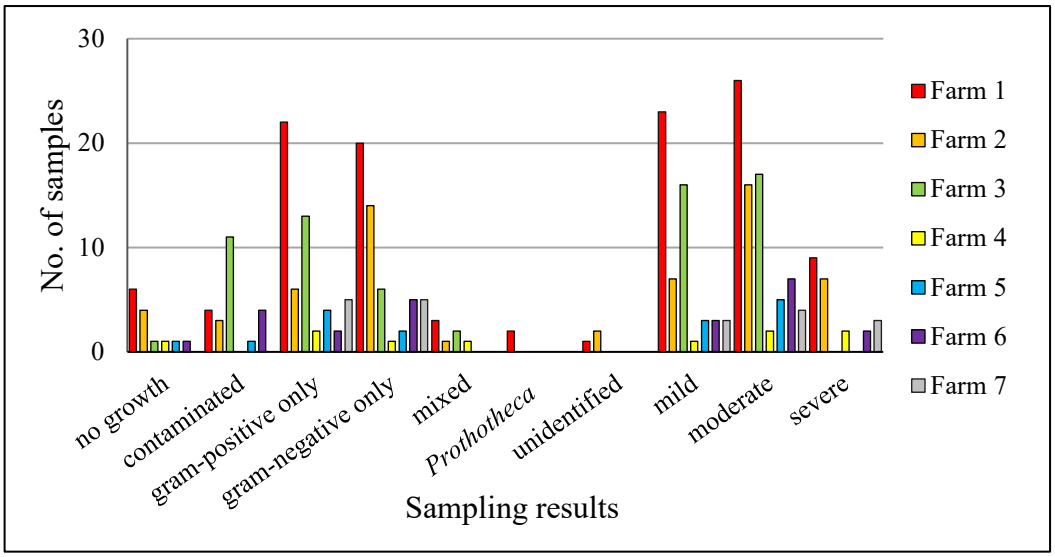
3.3.1 Reference test

The number of milk samples collected per farm ranged from 5 to 58 (**Table 3-1**). Out of 156 samples, 23 (14.7%) were contaminated. Among 133 non-contaminated samples, 14 (10.5%) showed no growth, and 116 (87.2%) showed growth of one or two colony types that could be identified by the reference method (**Table 3-3**). Three samples yielded growth of organisms that could not be identified by the reference method, and those samples were excluded from further analysis.

Within each farm's sample set, both gram-positive and gram-negative isolates were identified, with a preponderance of gram-positive results for some farms (Farms 3, 4 and 5; **Figure 3-2**), mostly gram-negative results for others (Farms 2 and 6; **Figure 3-2**) and an even balance for the remainder (Farms 1 and 7; **Figure 3-2**). The proportion of contaminated samples per farm ranged from 0 to 33%, indicating considerable differences in sample quality.

The most identified species were *E. coli* and *S. uberis*, followed by other common MCB, including *Strep. dysgalactiae*, *Staph. aureus* and *Klebsiella* (**Table 3-3**).

Figure 3-2 Sampling results for bovine milk samples (n = 130) from quarters with clinical mastitis by participating farm. The number of samples for each farm were from farm 1 to 7 respectively 58, 30, 35, 5, 8, 12 and 10.



3.3.2 Slide test

After excluding contaminated samples and samples that yielded non identifiable organisms, 130 milk samples were used to evaluate the performance of the slide test at milk sample level. A milk sample could be culture negative or culture positive, contain a single colony type or two colony types, i.e. both gram positive, both gram negative, or mixed gram-positive and gram negative. Mixed cultures were considered gram-positive in the gram-positive analysis and gram-negative in the gram-negative analysis. Based on these samples the proportion of culture negative results was considerably higher for the slide test (20%) than for the reference test (10.8%). Of 26 culture-negative samples in the slide test, 12 (46.2%) were correctly classified (**Table 3-3**). Of the 14 FN slide test results, 7 were from samples with gram-positive growth in the reference test and 7 from samples with gram-negative growth (**Table 3-3**). Mixed gram-positive and gram-negative growth was more common in the slide test (12.3%) than in the reference test (5.4%) (**Table 3-3**).

Table 3-3 Test results of 130 milk samples from bovine clinical mastitis based on a reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS) in comparison with the simplified slide test (VétoSlide, Vétoquinol, Lure, France) and a commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France).

Result	Reference Number[1] (%)	VétoSlide Number[1] (%)	VétoRapid Number[1] (%)
No growth	14 (10.8)	26 (20.0)	17 (13.1)
Growth	116 (89.2)	104 (80.0)	113 (86.9)
Gram-positive only	54 (41.5)	50 (38.5)	55 (42.3)
Gram-negative only	53 (40.8)	38 (29.2)	30 (23.1)
Mixed (gram-positive & gram-negative)	7 (5.4)	16 (12.3)	28 (21.5)
Gram-negative	60 (46.2)	54 (41.5)	58 (44.6)
Escherichia coli	51 (39.2)	45 (34.6)	50 (38.5)
Klebsiella spp.	6 (4.6)	n/a	16 (12.3)
Other gram-negative	4 (3.1)	n/a	2 (1.5)
Gram-positive	61 (46.9)	66 (50.8)	83 (63.8)
Staphylococcus spp.	22 (16.9)	n/a	56 (43.1)
Staphylococcus aureus	7 (5.4)	n/a	43 (33.1)
Non-aureus staphylococci	15 (11.5)	n/a	21 (16.2)
Streptococcus spp.	32 (24.6)	n/a	50 (38.5)
Streptococcus dysgalactiae	10 (7.7)	n/a	11 (8.5)
Streptococcus uberis	21 (16.2)	n/a	38 (29.2)
Other *Streptococcus* spp.	1 (0.8)	n/a	6 (4.6)
Enterococcus spp.	3 (2.3)	n/a	2 (1.5)
Other gram-positive	13 (10.0)	n/a	1 (0.8)
Prototheca zopfii	2 (1.5)	n/a	n/a

[1]The total number of species/genera listed exceeds the number of samples because more than one species/genus was detected in some samples that were not contaminated based on the NMC standard definition of 3 or more colony types (the percentage shown is related to the proportion of samples). n/a = not applicable.

The sensitivity of the slide test was similar for gram-positive and gram-negative organisms, but specificity was higher for the latter, resulting in higher accuracy for gram-negative organisms (89.2%) than for gram-positive organisms (79.2%), and higher yet for *E. coli* (accuracy 92.3%; **Table 3-4**). For the samples from non-severe CM cases (n = 109), the potential of the slide test to be used as treatment decision support tool was evaluated. Sensitivity and specificity for the subset of non-severe CM cases were similar to those for all CM cases (**Table 3-4**). Positive predictive value of the slide test was high (>92.6%) for growth, gram-negative results, and *E. coli* and moderate (between 75 and 80%) for gram-positive results and treatment. The negative predictive value was less than 50% for growth, but over 80% for all other outcomes (**Table 3-4**).

Table 3-4 Performance of the simplified slide test under evaluation (VétoSlide, Vétoquinol, Lure, France) and a commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) for identification of mastitis pathogens (n = 130 samples). Results are based on comparison with a reference test consisting of culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry and the difference represents the comparison of both tests when correcting for dependence. Values represent point estimates expressed as percentage with 95% confidence intervals in brackets.

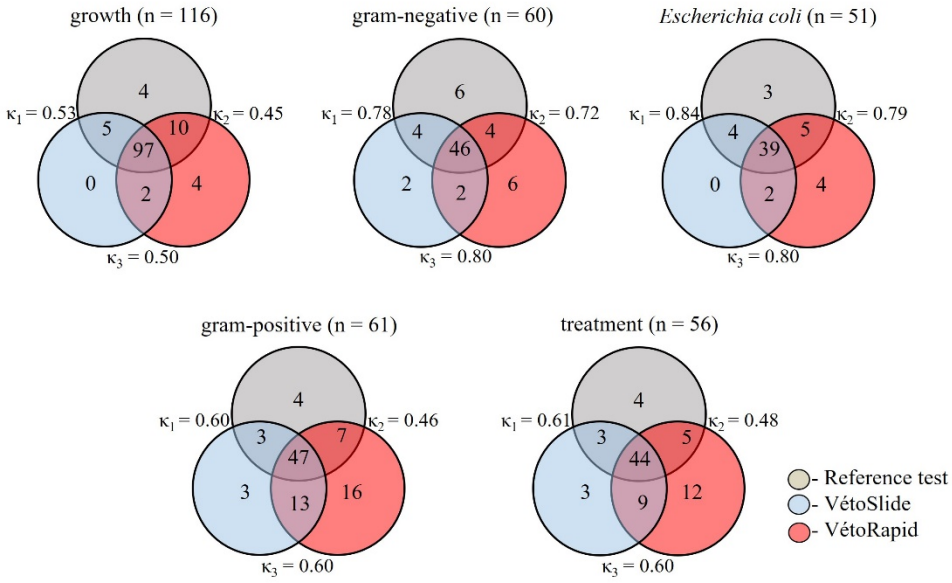
	VétoSlide	VétoRapid	Difference
Sensitivity			
Growth (n = 116)	87.9 [80.8; 92.7]	92.2 [85.9; 95.9]	4.3 [-2.2; 10.8]
Gram-negative (n = 60)	83.3 [72.0; 90.7]	83.3 [72.0; 90.7]	0.0 [-9.2; 9.2]
Gram-positive (n = 61)	82.0 [70.5; 89.6]	88.5 [78.2; 94.3]	6.6 [-3.5; 16.6]
E. coli (n = 51)	84.3 [72.0; 91.8]	86.3 [74.3; 93.2]	2.0 [-9.6; 13.5]
Treatment (n = 56)	83.9 [72.2; 91.3]	87.5 [76.4; 93.8]	3.6 [-6.3; 13.4]
Specificity			
Growth (n = 14)	85.7 [60.1; 96.0]	57.1 [32.6; 78.6]	-28.6 [-52.2; -4.9]*
Gram-negative (n = 70)	94.3 [86.2; 97.8]	88.6 [79.0; 94.1]	-5.7 [-13.5; 2.1]
Gram-positive (n = 69)	76.8 [65.6; 85.2]	58.0 [46.2; 68.9]	-18.2 [-30.4; -7.3]*
E. coli (n = 79)	97.5 [91.2; 99.3]	92.4 [84.4; 96.5]	-5.1 [-9.9; -0.2]*
Treatment (n = 53)	77.4 [64.5; 86.5]	60.4 [46.9; 72.4]	-17 [-30.6; -3.4]*
Accuracy			
Growth (n = 130)	87.7 [80.9; 92.3]	88.5 [81.8; 92.9]	0.8 [-5.8; 7.3]
Gram-negative (n = 130)	89.2 [82.7; 93.5]	86.2 [79.2; 91.1]	-3.1 [-9.1; 2.9]
Gram-positive (n = 130)	79.2 [71.5; 85.3]	72.3 [64.1; 79.3]	-6.9 [-15; 1.1]
E. coli (n = 130)	92.3 [86.4; 95.8]	90.0 [83.6; 94.1]	-2.3 [-7.7; 3.1]
Treatment (n = 109)	80.7 [72.3; 87.0]	74.3 [65.4; 81.6]	-6.4 [-15; 2.1]
Positive predictive value			
Growth	98.1 [93.3; 99.5]	94.7 [88.9; 97.5]	-3.4 [-6.7; -0.03]*
Gram-negative	92.6 [82.4; 97.1]	86.2 [75.1; 92.8]	-6.4 [-15.2; 2.4]
Gram-positive	75.8 [64.2; 84.5]	65.1 [54.3; 74.4]	-10.7 [-18.9; -2.55]*
E. coli	95.6 [85.2; 98.8]	88.0 [76.2; 94.4]	-7.6 [-14.8; -0.3]*
Treatment	79.7 [67.7; 88.0]	70.0 [58.5; 79.5]	-9.7 [-18.5; -0.86]*
Negative predictive value			
Growth	46.2 [28.8; 64.5]	47.1 [26.2; 69.0]	0.9 [-18.7; 20.5]
Gram-negative	86.8 [77.4; 92.7]	86.1 [76.3; 92.3]	-0.7 [-7.3; 5.8]
Gram-positive	82.8 [71.8; 90.1]	85.1 [72.3; 92.6]	2.3[-7.3; 11.9]
E. coli	90.6 [82.5; 95.2]	91.3 [83.0; 95.7]	0.7 [-5.8; 7.2]
Treatment	82.0 [69.2; 90.2]	82.1 [67.3; 91.0]	0.1 [-10.7; 10.8]

*Outcomes where VétoSlide and VétoSlide are significantly different.
The total of samples that yield in the reference test growth, Gram-negative, Gram-positive, *E. coli* and Treatment were, 116, 60, 61, 51 and 56, respectively.

3.3.3 Agreement between tests

The agreement between the slide test and the comparator test as expressed in Cohen's κ was only moderate (0.45 to 0.53). This was partly due to the predominance of culture positive results among the 130 non-contaminated samples, leaving limited room for agreement beyond chance (**Figure 3-3**). In comparisons against the reference test, the slide test outperformed the comparator test for detection of gram-positive organisms in non-severe mastitis cases, with substantial (κ = 0.61) and moderate (κ = 0.48) agreement respectively, and for detection of *E. coli* across all non-contaminated samples, with almost perfect (κ = 0.84) and substantial agreement (κ = 0.79), respectively.

Figure 3-3 Venn diagrams showing agreement between the reference test (top), simplified slide test (VétoSlide; bottom left), and commercially available plate-based comparator (VétoRapid; bottom right). Numbers represent the number of samples testing positive for each result, with the position in the Venn diagram showing which test(s) yielded that result. Numbers (n) in labels refer to results of the reference test. κ values indicate Cohen's kappa for agreement between two tests (κ_1 = agreement between VétoSlide and reference test; κ_2 = agreement between VétoRapid and reference test; κ_3 = agreement between VétoSlide and VétoRapid). The outcomes growth, gram-positive, gram-negative and Escherichia coli are based on non-contaminated samples with no growth or with identifiable pathogens from all cases of mastitis (n = 130). Result for treatment are based on non-contaminated samples with no growth or identifiable pathogens from non-severe cases of mastitis (n = 109).



3.3.4 Benchmarking against the comparator test

Despite using a higher inoculum volume than the comparator test, the slide test gave more false negative and fewer false positive results in terms of growth and had significantly greater specificity but similar sensitivity. Similar results were obtained for the outcomes Gram-positive, *E. coli* and treatment when comparing the slide test to the comparator test. The low specificity of the comparator test for growth, gram-positive and treatments was partly due to poor specificity in the detection of *Staph. aureus* and *S. uberis* (70.7% and 82.6%, respectively; **Table 3-5**), which was accompanied by high sensitivity for the same pathogens, as well as *E. coli* (100%, 90.5% and 86.3%, respectively). In terms of sensitivity, there were no statistically significant differences among tests for any of the outcomes (**Table 3-4**).

Despite the greater specificity of the slide test, overall accuracy of the two tests was not significantly different. In my study population, positive predictive value of the slide test was significantly greater than for the comparator test for growth, gram-positive, *E. coli* and treatment. Negative predictive values for the two tests were similar for all outcomes.

Table 3-5 Performance of the commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) for identification of mastitis pathogens (n = 130 samples) based on comparison of results at species, genus or group level with a reference test consisting of non-selective culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry. Values represent point estimate expressed as percentage with 95% CI in brackets.

	Gram-positive						Gram-negative		
Epidemiological parameter	*Staphylococcus aureus* (n = 7)	Non-aureus Staphylococci (n = 15)	*Streptococcus dysgalactiae* (n = 10)	*Streptococcus uberis* (n = 21)	*Streptococcus agalactiae* (n = 0)	*Enterococcus* spp. (n = 3)	*Escherichia coli* (n = 51)	*Klebsiella* spp. (n = 6)	Other gram-negative (n = 4)
True prevalence	5.4 [2.6; 10.7]	11.5 [7.1; 18.2]	7.7 [4.2; 13.6]	16.2 [10.8; 23.4]	0 [0; 2.9]	2.3 [0.8; 6.6]	39.2 [31.3; 47.8]	4.6 [2.1; 9.7]	3.1 [1.2; 7.6]
Apparent prevalence	33.1 [25.6; 41.5]	16.2 [10.8; 23.4]	8.5 [4.8; 14.5]	29.2 [22.1; 37.6]	4.6 [2.1; 9.7]	1.5 [0.4; 5.4]	38.5 [30.5; 47]	9.2 [5.4; 15.4]	0.8 [0; 4.2]
Accuracy	72.3 [64.1; 79.3]	81.5 [74.0; 87.3]	93.1 [87.4; 96.3]	83.8 [76.6; 89.2]	95.4 [90.3; 97.9]	96.2 [91.3; 98.3]	90.0 [83.6; 94.1]	92.3 [86.4; 95.8]	96.2 [91.3; 98.3]
Sensitivity	100 [64.6; 100]	40.0 [19.8; 64.3]	60.0 [31.3; 83.2]	90.5 [71.1; 97.3]	not applicable	0 [0; 56.1]	86.3 [74.3; 93.2]	66.7 [30.0; 90.3]	0 [0; 49.0]
Specificity	70.7 [62.2; 78.0]	87.0 [79.6; 91.9]	95.8 [90.6; 98.2]	82.6 [74.4; 88.5]	95.4 [90.3; 97.9]	98.4 [94.4; 99.6]	92.4 [84.4; 96.5]	93.5 [87.8; 96.7]	99.2 [95.6; 100]
Positive predictive value	16.3 [8.1; 30.0]	28.6 [13.8; 50.0]	54.5 [28.0; 78.7]	50.0 [34.8; 65.2]	0 [0; 39.0]	0 [0; 65.8]	88.0 [76.2; 94.4]	33.3 [13.8; 60.9]	0 [0; 94.9]
Negative predictive value	100 [95.8; 100]	91.7 [85.0; 95.6]	96.6 [91.7; 98.7]	97.8 [92.4; 99.4]	100 [97.0; 100]	97.7 [93.3; 99.2]	91.3 [83.0; 95.7]	98.3 [94.0; 99.5]	96.9 [92.3; 98.8]

3.4 Discussion

In the current study, I evaluated the laboratory performance of a simplified culture-based slide test, VétoSlide, which was developed as a potential point-of-care tool to support farmers' CM treatment decisions. The new test was also benchmarked against a commercially available plate-based comparator test that is currently used in my practice area. The simplified test outperformed the plate-based comparator test in specificity but not in sensitivity or overall accuracy. The simplicity of the slide test can make it an attractive tool for farmers to target antimicrobial treatment to cows with non-severe CM that yield growth or, more specifically, gram-positive culture results from milk samples.

The test was easy to perform it required only inoculation of a CM milk sample on pre-prepared media, making it suitable for use outside of a formal laboratory setting, in line with its original design for office-based urine dip-slide testing (Van Dorsten and Bannister, 1986). Based on the manufacturer's instructions, slides can be swabbed with milk, as done in my study, or dipped in milk. The universal vials that are routinely used for milk sample collection on the farms in my practice area are not large enough for the dip method, which is why I used the swab method. Alternatively, larger milk sampling containers could be used, but this would increase the risk of sample contamination. Even with current sampling methods, sample contamination, together with the willingness to collect samples, were identified as issues that need to be overcome for routine on-farm uptake of the test. Slide test results are easy to interpret based on the qualitative reading of growth on each side of the slide, which are differentiated by the colours red and green. For colour blind people this may pose a problem, as difficulty distinguishing between red and green is the most common form of colour blindness (Neitz and Neitz, 2000). For further evaluation of convenience criteria, on-farm studies with end-users should be conducted, as already done for Petrifilm (Mansion-de Vries *et al.*, 2014a) and the Minnesota easy culture System (Royster *et al.*, 2014a). Affordability is also considered part of the convenience criteria for test evaluation but should be considered in the context of cost-benefit, particularly in farming, which is an economic activity. The economic value of pathogen information in mastitis treatment depends on several factors, including the presence of a dominant pathogen species on the farm and the risk of pathogen transmission (Cha *et al.*, 2013; Down *et al.*, 2017). Likewise, the ability of on-farm diagnostics to reduce antimicrobial use depends on the prevalence of gram-positive and gram-negative organisms (Mansion-de Vries *et al.*, 2014a). In my study, some farms

had a predominant pathogen type whereas others did not. Therefore, the value of information would be farm-specific and no blanket statements around cost-benefit or reductions in antimicrobial use cannot be made based on my results. It is clear, however, that some farms will need further training in sample collection and handling to reduce the number of contaminated samples and make investment of time and money into diagnostic testing better value for money.

The accuracy of gram-positive detection in samples from non-severe CM was 80.7% with the slide test (VétoSlide), placing it in the same range as commercially available point-of-care tests, including the comparator test (VétoRapid) (74.3%, this study), the Minnesota Easy Culture System-Triplate (81.3%) (Ferreira *et al.*, 2018), Minnesota Easy Culture System-biplate (81 to 84%) (Royster *et al.*, 2014a), Petrifilm (80.2%) (Mansion-de Vries *et al.*, 2014a) and MastDecide (58.6 to 85.3%) (Leimbach and Krömker, 2018). However, when comparing performance results of the present tests to other studies, caution is required as study designs (e.g. definitions of intramammary infections and reference standards), study populations (animal and pathogen populations), and methods to calculate confidence intervals differ. For example, *Staph. chromogenes* was not detected among the NAS species in this study, which is surprising because it is the most common NAS in most other studies (Zadoks and Watts, 2009).

In practice, the positive and negative predictive value of a test are more important than the sensitivity, specificity or accuracy, but both depend on pathogen prevalence in the population, making them study specific. For that reason, I benchmarked the slide test against the comparator test in a single study, allowing for direct comparison of predictive values. Positive predictive values were higher for the slide test, implying that it was less likely to result in unnecessary treatment than use of the comparator test. Negative predictive values for the slide test were similar to those of the comparator test, meaning that the reduced risk of over-treating was not accompanied by an increased risk of under-treating. Failure to treat based on false negative results for gram-positive organisms may negatively impact cow welfare whereas false positive results for gram-positive organisms will lead to a sub-optimal reduction of antimicrobial use. Whether positive or negative predictive value is considered more important in informing treatment decisions differs between regions. In some areas, such as southern Europe (Busani *et al.*, 2004), it is generally assumed that antimicrobial treatment of mastitis is needed until proven otherwise, in agreement with my experience of

working in the area. Conversely, in northern Europe, it is assumed that treatment is not needed until proven otherwise (Jørgensen *et al.*, 2016; Persson Waller *et al.*, 2016). Within countries, this balance may shift over time, as illustrated by work from The Netherlands on selective DCT. Two split-udder trials conducted two decades apart (Schukken *et al.*, 1993; Scherpenzeel *et al.*, 2014) in the same country both showed that blanket DCT prevents CM when compared to selective DCT. However, the first study concluded that blanket DCT should be used to prevent CM despite the need to "eliminate unnecessary use of antibiotics"; whereas the second study emphasized the reduction in antimicrobial use that could be achieved by abandoning blanket DCT. This illustrates how similar outcomes may be presented differently depending on shifts in societal concerns. The number of on-farm diagnostics described and available in North America to inform lactational treatment of CM suggests a similar shift in attitude away from blanket treatment to targeted or selective (McCarron *et al.*, 2009a; Lago *et al.*, 2018).

In the current study, frozen milk samples thawed under ambient temperature rather than fresh milk samples were used. According to some authors, freezing of milk samples may increase the frequency of isolation of *Strep. agalactiae* and *Staph. aureus* (Villanueva *et al.*, 1991; Sol *et al.*, 2002) whereas others report no impact on recovery of *Streptococcus* spp. or *Staph. aureus* (Schukken *et al.*, 1989) or even a decrease in recovery of *Strep. agalactiae* and *Strep. dysgalactiae* (Sol *et al*., 2002). Likewise, some authors report that freezing may affect recovery of *E. coli* (Schukken *et al.*, 1989) whilst others found no effect of freezing for 6 weeks on *E. coli* viability (Murdough *et al.*, 1996). Although freezing may have affected the prevalence of certain isolates recovered in this study, it should not have affected the comparison of results between the reference test, the slide test, and the commercially available comparator plate-based test.

Thawing of milk samples at room temperature for up to 8 hours may have influenced my results by acting as a pre-incubation step. Some authors found that pre-incubation enhances the detection of *Staph. aureus* but not streptococci (Sol *et al.*, 2002), which contrasts with results from others, who reported increased detection of streptococci and coliforms after pre-incubation (Dinsmore *et al.*, 1992). Published pre-incubation studies focus on incubation at 37ºC rather than room temperature. Even at 37ºC, pre-incubation for 4 hours does not lead to increased detection of contamination (Dinsmore *et al.*, 1992) so the relatively high level of contamination in my study is likely to be the result of contamination

during sample collection rather than my culture method. The farm-specific level of contamination supports this notion.

A larger volume of milk was inoculated on the slide test than on the reference test or the commercial comparator test. An increase in sample volume enhances bacterial recovery (Dinsmore *et al.*, 1992), which could translate into higher sensitivity (fewer false negatives) as well as lower specificity (more false positives). In this study, no such effect was observed, as sensitivity was not different between slide test and comparator test while specificity was higher for the slide test, despite the use of a higher inoculum volume. The reference test used in this study include species identification by MALDI-ToF MS and revealed the presence of several species that are not recognized as typical mastitis pathogens, e.g. *Bacillus* and *Lysinibacillus* species. Although both genera are gram-positive, it is debatable whether they should be targeted with antimicrobial treatment because little is known about their role as pathogenic agents or their response to treatment. None of the currently available point-of-care tests for mastitis have the ability to differentiate such organisms from recognized mastitis pathogens, and conventional microbial diagnostics continue to be important for the identification of pathogens to species level (Mansion-de Vries *et al.*, 2014a). For three isolates, species identification was not possible, demonstrating the limitations of MALDI-ToF MS as it only identifies microbial spectra that are available in the database (Cameron *et al.*, 2017).

Finally, the value of knowledge and diagnostic information depends on their implementation. Even if farmers acknowledge the existence of management practices that would benefit their farm management, they often do not implement them (Barkema, Schukken, *et al.*, 1999). Farmers often experience “insecurity” about how to treat mastitis and “uncertainty” about treatment efficacy or cow recovery after treatment (Jansen and Lam, 2012; Swinkels *et al.*, 2015). Point-of-care tests can reduce this insecurity and allow for the implementation of evidence-based treatment approaches. Whilst a detailed result, namely pathogen identification, as provided by the comparator test (VétoRapid) and other tests comprising three or more selective indicator media, is the preferred outcome for diagnostic tests for subclinical mastitis, it is not farmers’ priority for CM diagnostics (Griffioen *et al.*, 2016). For more detail on social aspects of diagnostic test use please see Chapter **5**. General discussion.

Chapter 4. Exploration of a culture-independent biomarker as indicator to inform targeted mastitis treatment

4.1 Introduction

Antimicrobial resistance (**AMR**) constitutes an important global public health problem and, in some parts of the world, also an animal health problem. Antimicrobial use (**AMU**) contributes to the emergence of this problem (O'Neill, 2014). To reduce AMU in the dairy sector, several strategies have been proposed. Targeted treatment of CM can promote prudent AMU without jeopardizing animal health and welfare. There are multiple diagnostic tests to support treatment decisions, with different advantages and limitations (Malcata *et al.*, 2020).

Currently, there are many diagnostic tools that are culture-based. They offer the advantage of being able to be used on-farm as point-of-care tests and can identify the pathogen. Because they depend on culture, they have long turn-around times. In addition, these tests often require user training and experience, with user training needed both for test interpretation and sample collection (see: **Chapter 3**). Otherwise, sample contaminants might indicate that treatment is needed and may result in a sub-optimal reduction of AMU.

In theory, culture-independent tests could offer shorter turn-around times. They can identify the pathogen or even target AMR genes based on DNA methods, for example using PCR or LAMP. However, these methods have a higher cost than culture when conducted off-farm and are not yet available for on-farm use. Other culture-independent tests can detect inflammation instead of the pathogen, which is used as a proxy for infection. Advances in proteomic techniques allowed the identification of multiple mastitis biomarkers such as haptoglobin (Åkerstedt *et al.*, 2009), serum amyloid A (Eckersall *et al.*, 2006), N-acetyl-β-D-glucosaminidase (Pyörälä *et al.*, 2011), lipopolysaccharide-binding protein (Schroedl *et al.*, 2001) and, more recently, cathelicidins (Addis *et al.*, 2016, 2017; Wollowski *et al.*, 2021). However, they lack pathogen-specificity, and may not be able to support treatment decisions.

Several pathogen-associated molecular patterns are recognised in the udder by pattern recognition receptors that instigate a cascade of events leading to the release and expression of antimicrobial molecules and cytokines from host cells in the mammary gland (Günther *et al.*, 2017; Petzl *et al.*, 2018). There is evidence that host response is specifically adapted to different mastitis-causing pathogens and strains (Bannerman *et al.*, 2004; Blum *et al.*, 2017). Therefore, host-response biomarkers that are pathogen-specific could potentially be used to inform treatment decisions. In comparison with culture-based tests, they would have the advantage that they provide a diagnosis in minutes or hours. There is some promising evidence that supports this approach, such as chemokines and cytokines that measured different immune response to gram-positive and gram-negative mastitis (Mansor *et al.*, 2013; Kusebauch *et al.*, 2018), or gene expression in udder tissue likely to be associated with pathogen and duration of infection (Loor *et al.*, 2011; Kościuczuk *et al.*, 2012). However, it is not always clear whether biomarker profiles reflect the severity of inflammation or the causative agents and concerns exist about the sensitivity and specificity of acute-phase proteins (Pyörälä *et al.*, 2011) or antimicrobial peptides such as cathelicidins (Addis *et al.*, 2016; Pongthaisong *et al.*, 2016; Wollowski *et al.*, 2021).

Cathelicidins are part of the host defence mechanism and have antimicrobial activity against different types of pathogens or potent chemotactic and proinflammatory functions that are able to neutralize infectious agents (Zanetti, 2004; Tomasinsig *et al.*, 2010; Young-Speirs *et al.*, 2018). These peptides are produced locally in mucosal epithelia or produced systemically and stored as preformed protein precursor in the leukocyte's cytoplasm. Cathelicidin production and release are triggered by microbial invasion, which can occur after an IMI and can be detected in milk (Zanetti, 2004; Cubeddu *et al.*, 2017). Recent research on these peptides gave them extra relevance in the development of new antimicrobial drugs and potential application in mastitis diagnostics (Young-Speirs *et al.*, 2018). They are highly correlated with SCC, can be eliminated in milk before leukocyte influx and SCC rise is detected, and are generally not detected in milk produced by healthy quarters. Therefore, they are recognized as highly sensitive and specific mastitis biomarkers (Smolenski *et al.*, 2014; Pongthaisong *et al.* 2015; Addis *et al.*, 2016).

Studies *in vivo* and *in vitro* allowed the discovery of seven different bovine cathelicidins molecules, with variable structures, modes of action and distinct antimicrobial properties (Selsted *et al.*, 1992; Scocchi *et al.*, 1997; Young-Speirs *et al.*, 2018). Proteomic

profile studies in naturally occurring CM and in experimentally induced infections by different bacteria showed different fold increases in the concentration of the seven cathelicidins between different studies and causative pathogens. Cathelicidin-1, -2, -3 and -4 have been detected in studies in infections with gram-positive and gram-negative bacteria (Ibeagha-Awemu *et al.*, 2010; Huang *et al.*, 2014; Mudaliar *et al.*, 2016), however, cathelicidin-5, -6 and -7 have been associated with gram-positive mastitis only (Reinhardt *et al.*, 2013; Huang *et al.*, 2014; Mudaliar *et al.*, 2016). Cathelicidin-2 was detected in severe CM but not in moderate CM cases caused by *E. coli* (Yang *et al.*, 2015).

A study examined the abundance of cathelicidin in an experimentally induced IMI using *S. uberis* as well in naturally occurring mastitis caused by diverse pathogens, found that cathelicidin increases after IMI, to be correlated to SCC, but its abundance may vary for different stages of infection or different clinical presentations (Smolenski *et al.*, 2011). In another study, cathelicidin median levels were particularly increased in mastitis caused by *Strep. agalactiae*, followed by *Staph. aureus*, and non-agalactiae streptococci. Clinical mastitis caused by gram-negative bacteria, or coagulase negative staphylococci and culture negative CM were associated with lower median cathelicidin values (Addis *et al.*, 2017). Despite the variations in cathelicidin concentrations, it was concluded that cathelicidin levels did not show enough discriminatory power to guide treatment decisions. Neither Smolesnki *et al.* (2011) nor Addis *et al.* (2017) described severity levels of CM. A study that included both severity levels and CM-causing pathogen information demonstrated that cathelicidin abundance in CM cases was not influenced by severity or the bacteriological result, despite reporting some significant differences in cathelicidin levels in quarters with SCM caused by coagulase negative Staphylococci, *Staph. aureus* and *S. uberis* (Wollowski *et al.*, 2021). All these studies agree that cathelicidin abundance is not the same for all mastitis cases. However, is still not clear whether these variations reflect the severity of inflammation or the MCB or other potential factors. In all these studies, bacteriological identification with broad categories, to differentiate gam-positive CM from other CM cases was never done and in most of these studies, information about CM severity was lacking, which are known as relevant and enough to inform CM treatment decisions (Malcata *et al.*, 2020).

Based on this knowledge gap, the aim of this study was to investigate if a culture-independent biomarker, cathelicidin, can be used to differentiate mild-to-moderate gram-

positive CM from other forms of CM, which includes severe CM and/or CM caused by non-gram-positive pathogens.

4.2 Material and Methods

4.2.1 Regulatory compliance

This research was approved by the Ethics and Welfare Committee of the School of Veterinary Medicine of The University of Glasgow, UK (Ref 50a/16).

4.2.2 Sample collection

Seven dairy farms in Scotland were selected for participation based on herd size, location, and willingness to cooperate in the study (for participating farms' information please refer to Table 3-1 in section 3.2. Material and Methods). Farm staff, including milkers and herd managers, were recruited to participate in the study and trained by me on how to identify CM cases, how to classify each CM case for severity, and how to collect milk samples aseptically as explained in **Chapter 3**. One farmer was asked to sample additional milk samples from clinically healthy cows, with no signs of CM and SCC < 200.000 cells/mL.

Samples were collected in two periods: from January to May 2018 and from September to November 2020. The first sampling period included seven farms, the second sampling period included just 4 farms, based on farm location and willingness to cooperate in the study.

Samples were stored frozen on-farm (-20°C), and once a week I transported them to Glasgow University's Veterinary Diagnostic Services laboratory where they were stored frozen (-20°C) until processing. All samples were cultured by me within 4 weeks from CM detection.

4.2.3 Milk microbiology

Samples were thawed at ambient temperature for up to 8 hours and were processed using standard bacteriological culture methods (NMC, 2017), with subsequent determination of species identity using MALDI-ToF MS as the reference test. Sheep blood agar (5% vol/vol; SBA) and MacConkey agar number 3 plates (E&O Laboratories Limited, Bonnybridge, Scotland) were inoculated with 0.01 mL of milk each using disposable sterile calibrated plastic loops. After processing the plates for bacteriology aliquots from the milk samples were taken for molecular biology and stored frozen (-20°C). Plates were incubated at 37°C in aerobic conditions and examined after approximately 48 hours. Samples that did not yield growth of visible colonies were considered negative for mastitis-associated pathogens. Samples that yielded three or more colony types were considered contaminated and excluded from data analysis in accordance with NMC guidelines. For the remaining plates, each morphotype was identified by standard laboratory methods (NMC, 2017) including colony morphology, growth on MacConkey, catalase test, haemolysis, coagulase test and gram-staining. For each non-contaminated sample, each colony type was sub-cultured onto half of an SBA plate for purification. From each pure culture, a colony was selected and grown in 2 mL of Brain Heart Infusion broth for 24 hours at 37°C in aerobic conditions without shaking. The isolates were preserved with 15% glycerol (v/v) in cryovials at -80°C and submitted to two external laboratories for species identification by MALDI-ToF MS. Isolates from the first sampling period, January to May 2018, were submitted to the Laboratoire de Microbiologie, (Vétoquinol SA, Lure, France) and identified using Vitek-MS and V3.1.0 database (bioMérieux, Marcy-l'Étoile, France). Isolates from the second sampling period were submitted to the Laboratory of Quality Milk Management Services (QMMS, Easton, Wells, Somerset, United Kingdom) and identified using Microflex - Flex Control Version 3.4 (Bruker Daltonics, Billerica, Massachusetts, USA) and MBT IVD (DB-5989 MSP) and MBT Compass (DB-5989 MSP) databases, respectively.

4.2.4 Milk molecular biology – cathelicidin measurement

4.2.4.1 Protein quantification

Total protein concentrations for a subset of CM samples with no growth, or growth of one identifiable isolate, as gram-positive or gram-negative, and all the healthy quarter

milk samples that were not contaminated were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Life Science Ltd, UK). Aliquots of the original milk samples taken for bacteriological culture were thawed at ambient temperature for less than 1 hour and subsequently homogenized on a vortexer. Each sample was diluted in purified water (Milli-Q^{TM}) at a proportion of 1/40 (v/v), using 10 µL of milk and 390 µL of purified water. BCA standards were prepared using the same diluent as the samples in accordance with the manual of the Pierce BCA Protein Assay Kit (Thermo Life Science Ltd, UK) (**Table 4-1**). The buffer solution was created by mixing Pierce BCA protein assay sample Reagent A with Reagent B (1/40) prior to adding to each well. Each diluted sample (25 µL) and standards (25 µL) were pipetted into the appropriate wells on the microplate, with 200 µL of buffer solution. The microplate was agitated at 350 rotations per minute (rpm) on an orbital microplate shaker for 30 seconds and incubated for 30 minutes at 37°C. After incubation, the plate was cooled at room temperature for 3 minutes and then read at 562 nm on a microplate reader, using Ascent software (Multiskan Ascent, MTX Lab Systems). Data was analysed using Microsoft Excel, (2010) and GraphPad Prism software, version 5 for Windows (GraphPad Software, San Diego, California, USA. The average 562 nm absorbance measurement of the blank standard was subtracted from the 562 nm absorbance measurements of all other standards and unknown samples, and a standard curve was generated by plotting the average blank-corrected 562 nm measurement for each BSA protein standard against its concentration in µg/mL. Comparison of absorbance against this standard curve allowed the determination of the protein concentration of each sample.

Table 4-1 Preparation of diluted BSA standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working range = 20-2,000 µg/mL)			
Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	150 of Stock	2000
B	62.5	187.5 of Stock	1500
C	162.5	162.5 of Stock	1000
D	87.5	87.5 of Vial B dilution	750
E	162.5	162.5 of Vial C dilution	500
F	162.5	162.5 of Vial E dilution	250
G	162.5	162.5 of vial F dilution	125
H	200	50 of vial G dilution	25
I	200	0	0=Blank

4.2.4.2 Detection of cathelicidin presence in clinical mastitis milk samples

The cathelicidin presence was assessed using two Western blot techniques (A and B) using aliquots from original CM milk samples. For these assays, a commercial human primary polyclonal antibody was used, i.e. anti-human cathelicidin (Antibody 16135, HuCal BioRad Ltd, UK), which had previously been investigated, along with 14 other human primary anti-human cathelicidin antibodies, in Glasgow University's Proteomics laboratory (N. Brady personal communication). To produce these antibodies, a CTHL2-7_Pep1_Transferrin peptide (SSEANLYRLLELD-Ttds-C) was used because it was common to all seven bovine cathelicidins (Smolenski *et al.*, 2011). Therefore, these antibodies are polyclonal against this region and should have cross-reactivity towards all bovine cathelicidins. Antibody 16135 was selected because it generated the cleanest and most consistent signal at the expected molecular weights (17 and 28 kDa) with minimum nonspecific background, using aliquots from bovine CM samples from this project.

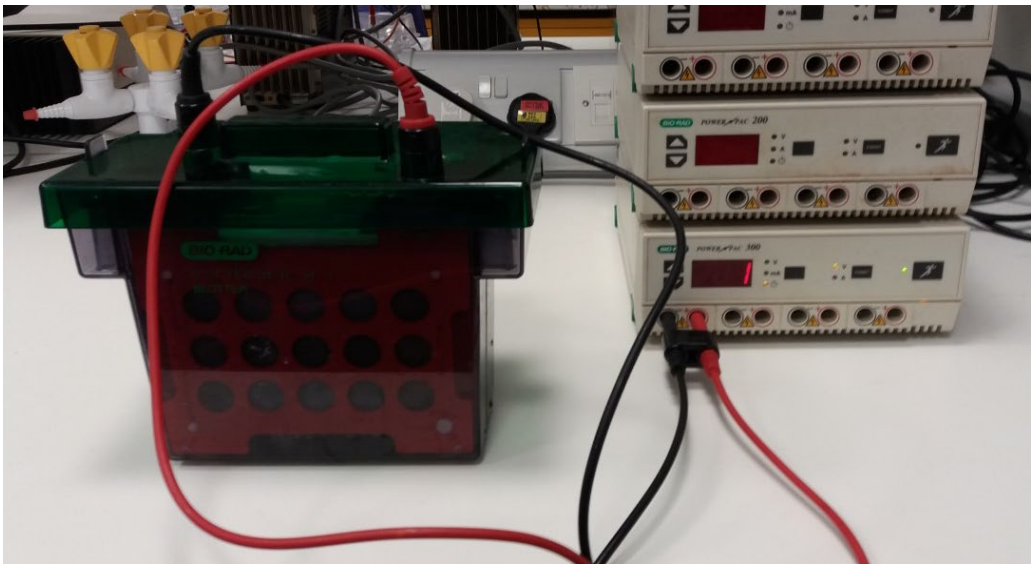
4.2.4.2.1 Western blot technique A

This method was previously described in bovine milk (Smolenski *et al.*, 2011) and was implemented here with slight modifications. Samples from CM cases where the culture results were known (culture negative samples and samples that yield a single morphotype that was identified as gram-positive or gram-negative) were chosen to evaluate my biomarker. Aliquots of milk samples were diluted to 2 mg/mL of protein with purified water (Milli-Q^{TM}) to give a sample volume of 50 µL. Diluted samples were mixed 1:1 with sample buffer. To prepare a 1:1 (vol/vol) mixture of diluted milk and sample buffer, 50 µL of sample buffer (prepared by combining 950 µL of 2x Laemmli sample buffer (BioRad Ltd, UK) and 50 µL β-mercaptoethanol) was mixed with 50 µL of diluted milk (2 mg protein/mL). Samples were vortexed for 6 seconds at 9000 rpm, incubated at 95°C for 4 minutes, and 15 µL were loaded on an 18-well gel (Criterion TGX precast midi protein gel, BioRad Ltd, UK).

In the first well, 7.5 µL protein molecular weight ladder (PageRuler Plus prestained ladder, ThermoFisher Scientific Ltd, Renfrew, UK) was added on its own in the gel. Samples were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in gel running buffer (10x Tris/Glycine/SDS buffer Ltd, UK; diluted 1:10 with distilled water) at 300 V for 15-20 minutes. After running the gel, it was blotted to

nitrocellulose membranes using an electrophoretic transfer at 70 V for 60 minutes through a blotting tank (**Figure 4-1**) and adding ice packs to the tank to keep the transfer cool while running.

Figure 4-1 Electrophoretic transfer using a blotting tank.



After the transfer, the membranes were blocked overnight at 4°C in 5% semi-skimmed milk solution, with Tris-buffered saline (pH 7.4) containing 0.5% Tween 20 (TBS-T), at 300 rpm on an orbital microplate shaker. Blots were washed three times for 10 min at a time with 0.5% TBS-T solution and then incubated with the primary antibody diluted 1:1587 in 1% semi-skimmed milk solution with TBS-T, with gentle shaking at 300 rpm on an orbital microplate shaker at room temperature for 60 min.

After incubation with the primary antibody, blots were washed three times for 10 minutes at a time with 0.5% TBS-T solution. Blots were then incubated with a secondary antibody, i.e. Goat anti-Human IgG F(ab')2 antibody (BioRad Ltd, UK) diluted 1:5000 in 1% semi-skimmed milk solution with TBS-T, with gentle shaking at 300 rpm on an orbital microplate shaker at room temperature for 60 min. After the incubation, the triple wash was repeated.

4.2.4.2.2 Western blot technique B and optimisation

This technique was previously used in canine cerebrospinal fluid proteomics (Shafie *et al.*, 2013) and adapted to milk samples with some modifications as described here. Sample selection criteria were similar to those for technique A, however, the sample set differed in terms of size and proportions of gram-positive and other than gram-positive samples, i.e. gram-negative bacteria and culture-negative samples. The technique used milk samples that were prepared as described under **4.2.4.2.1** Western blot technique A, by increasing concentrations of β-mercaptoethanol by 25%, 50%, 75% and 100% before addition to the sample buffer. An increase of 100% implies that twice the baseline concentration of β-mercaptoethanol was used. Samples were homogenized for 6 seconds at 9000 rpm by vortexing, incubated at 95°C for 4 minutes as previous (4.2.4.2.1 Western blot technique A), and by increasing heating times by 25%, 50%, 75% and 100%, i.e. 5, 6, 7 and 8 minutes. Then 15 and 10 µL of the 1:1 mixture of the sample with sample buffer were loaded on a 12 and 15 well gel (NuPAGE Novex 4-12% Bis-Tris Protein Gels, Invitrogen, ThermoFisher, Scientific Ltd, Renfrew, UK), using a ladder as described in technique A, a positive and negative controls, respectively, in each of the three first wells of the gel.

Electrophoresis was run in an XCell SureLock (Invitrogen, ThermoFisher Scientific Ltd, Renfrew, UK) electrophoresis tank using gel running buffer (NuPAGE MES SDS Running Buffer, Invitrogen, ThermoFisher Scientific Ltd, Renfrew, UK) 1:20 with distilled water at 150 V for 45 min (**Figure 4-2**).

Figure 4-2 Protein gel electrophoresis using 15-well gel.

After the electrophoresis, the gel was blotted to nitrocellulose membranes using a dry electrophoretic iBlot^{TM} chamber (Invitrogen, ThermoFisher Scientific Ltd, Renfrew, UK) (**Figure 4-3**).

Figure 4-3 Electrophoretic transfer using semi-dry blotting chamber.

Selected gels were run and stained with Coomassie Brilliant Blue, to allow visualisation of protein bands and confirm loading consistency across wells. When outliers were noticed, such as the absence of protein bands or unequal size of protein bands the SDS-PAGE process was repeated.

After the transfer, all the membranes were stained with Ponceau S to allow visualisation of protein bands and to confirm equal loading across wells and transfer consistency across the electrophoresis tracks. When outliers were apparent, lack of sample migration from the gel to the membrane, the correspondent samples were repeated or not considered for the analysis.

After de-staining (three 10-minute washes with 0.5% TBS-T), the blots were blocked at room temperature for 60 minutes using 5% semi-skimmed milk solution, with Tris-buffered saline (pH 7.4) containing 0.5% Tween 20 (TBS-T), at 300 rpm on an orbital microplate shaker. Blots were washed 3 times as described and incubated with anti-human cathelicidin diluted 1:1587, 1:3000, 1:5000 and 1:10000 in 1% semi-skimmed milk solution with TBS-T, with gentle shaking at 300 rpm on an orbital microplate shaker at 4°C for 60 minutes. After incubation with the primary antibody, washing was repeated as before and blots were then incubated with goat anti-human antibody (BioRad Ltd, UK) diluted 1:5000 in 1% semi-skimmed milk solution with TBS-T, with gentle shaking at 300 rpm on an orbital microplate shaker at room temperature for 60 minutes. After the incubation with the

secondary antibody the blots were washed again every 10 minutes for three times with 0.5% TBS-T solution.

4.2.4.2.3 Enhanced Chemiluminescence and film development

This procedure was the same for both techniques A and B. To detect the immunocomplexes, the enhanced chemiluminescence (ECL) reagent (Pierce ECL, ThermoFisher Scientific Ltd, Renfrew, UK) was prepared according to manufacturer's guidelines, mixing equal volumes of ECL reagents A and B. Then the nitrocellulose membrane was immersed in ECL solution and incubated for 5 min. After that, the ECL reagent was drained and the membrane was gently blotted with 3MM paper to remove the excess of ECL. Then, the membrane was wrapped in cling film and placed inside of a radiographic cassette.

To determine optimal exposure times for radiographic films, different exposure times were selected and trialled. Films were exposed to the blot, following ECL contact, for 30 seconds, 3 minutes and 5 minutes. Protein band density and background interference were assessed on radiographic films to determine the optimal exposure times and interpreted as presence or absence of cathelicidin in the corresponding CM sample.

4.2.5 Data analysis

Protein quantification results (response variable) were analysed using repeated-measures ANOVA, at the genus and species level, for broad categories, such as growth and gram-levels, and by severity level groups (explanatory variables). Pairwise t-test analysis using Bonferroni-error correction to adjust for multiple comparisons was employed using the Anova package in R (Harrell Jr and Dupont, 2019).

To evaluate cathelicidin performance, the reference test results were used to classify CM with presence of bacterial growth or presence of gram-positive bacteria and compared with western blot results for presence or absence of cathelicidin. Based on this comparison, cathelicidin results were classified as true positives (TP), true negatives (TN), false positives (FP) and false negatives (FP) as follows: CM samples with bacterial growth in the reference test and presence of cathelicidin in the western blot were considered TP and if cathelicidin was absent, they were considered FN. For samples that yielded no growth, if samples yielded

cathelicidin bands in the western blot they were considered FP, or TN if cathelicidin was absent in western blot. Likewise, gram-positive cases in the reference test with the presence of cathelicidin in the western blot were considered TP and if cathelicidin was absent, they were considered FN. For samples that yielded other results than gram-positive, i.e. gram-negative or culture-negative, if samples yielded cathelicidin bands in the western blot they were considered FP, or TN if cathelicidin was absent in western blot. From those classifications, sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows: Se = TP/(TP+FN), Sp = TN/(FP+TN), Ac = (TP+TN)/n, PPV = TP/(TP+FP), NPV = TN/(FN+TN).

To evaluate the potential of the culture-independent biomarker to support treatment decision, the calculations were repeated using a subset of samples, originated from non-severe CM cases only, that yielded gram-positive results in the reference test. For this comparison, the outcome was expressed as "treatment".

Biomarker performance statistical analysis was performed in Excel (Microsoft Corp., Redmond, USA) using tabular methods and in R (R Core Team, 2019). Epidemiological parameters were expressed as percentages with 95% Wilson type confidence intervals (CI), calculated using the Hmisc package in R (Harrell Jr and Dupont, 2019). Statistical significance was declared at $P < 0.05$. Agreement was expressed as Cohen's kappa coefficient (κ) and considered almost perfect ($\kappa > 0.8$), substantial ($0.61 \leq \kappa \leq 0.8$), moderate ($0.41 \leq \kappa \leq 0.6$), fair ($0.2 \leq \kappa \leq 0.4$), or slight ($\kappa < 0.2$) (Dohoo *et al.*, 2009).

4.3 Results

4.3.1 Milk sample microbiology

A total of 208 milk samples were collected across both periods (156 from January to May 2018, and 52 from September to November 2020). In total, 31 (14.9%) samples were contaminated, and three samples yielded growth but could not be identified by the reference method, therefore they were excluded from the study. From the remaining 174 non-contaminated samples 24 (13.8%) samples showed no growth, and 150 (86.2%) showed growth of one or two colony types that could be identified by the reference method. From the total sample set, proportions of gram-positive were smaller than gram-negative were

similar (42.5 vs 48.9%). At the species level, the most identified species were *E. coli*, and *S. uberis*, followed by non-aureus Staphylococci, *Klebsiella* spp. and *Strep. dysgalactiae*. Comparing the results of first and second sampling periods, the second period had a higher proportion of samples that yielded no growth, and fewer samples that yielded gram-positive results (**Table 4-2**).

Table 4-2 Test results of 177 milk samples from bovine clinical mastitis based on a reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Maldi-ToF MS)

Result	1st period[1] (%) (n = 130)	2nd period[1] (%) (n = 44)	Total sample set[1] (%) (n = 174)
No growth	14 (10.8)	10 (22.7)	24 (13.8)
Growth	116 (89.2)	34 (72.3)	150 (86.2)
Gram-positive only	54 (41.5)	9 (20.5)	63 (36.2)
Gram-negative only	53 (40.8)	21 (47.7)	74 (42.5)
Mixed (gram-positive & gram-negative)	7 (5.4)	4 (9.1)	11 (6.3)
Gram-negative	60 (46.2)	25 (56.8)	85 (48.9)
Escherichia coli	51 (39.2)	16 (36.4)	67 (38.5)
Klebsiella spp.	6 (4.6)	9 (20.5)	15 (8.6)
Other gram-negative	4 (3.1)	0 (0)	4 (2.3)
Gram-positive	61 (46.9)	13 (29.5)	74 (42.5)
Staphylococcus spp.	22 (16.2)	7 (15.9)	29 (16.7)
Staphylococcus aureus	7 (5.4)	1 (2.3)	8 (4.6)
Non-aureus staphylococci	15 (11.5)	6 (13.6)	21 (12.0)
Streptococcus spp.	32 (24.6)	4 (9.1)	36 (20.7)
Streptococcus dysgalactiae	10 (7.7)	0 (0)	10 (5.7)
Streptococcus uberis	21 (16.2)	3 (6.8)	24 (13.8)
Other *Streptococcus* spp.	1 (0.8)	0 (0)	1 (0.6)
Enterococcus spp.	3 (2.3)	1 (2.3)	4 (2.3)
Other gram-positive	13 (10.0)	1 (2.3)	14 (8.0)
Prototheca zopfii	2 (1.5)	0 (0)	2 (1.1)

[1]The total number of species/genera listed exceeds the number of samples because more than one species/genus was detected in some samples that were not contaminated based on the NMC standard definition of 3 or more colony types (the percentage shown is related to the proportion of samples).

When assessing sampling results at farm level, both gram-positive and gram-negative isolates were identified in each farm, with variations between farms between the two periods. Some farms had a preponderance of gram-positive results (Farms 4 and 5; **Table 4-3**), others had a preponderance of gram-negative results (Farms 2 and 6; **Table 4-3**) and the remaining farms an even balance between gram-positive and gram-negative (Farms 1, 3 and 7; **Table 4-3**) in the first sampling period. The proportion of contaminated samples per farm ranged from 0 to 33%, indicating heterogeneity in the quality of sampling (Table 4-3). When comparing the two sampling periods, for farms that participated in both periods, sample quality improved for Farm 3 but worsened for the other farms (Farms 1, 2 and 5).

Some farms showed a shift towards gram-negative results (Farms 1, 3). On the other farms (Farms 2 and 5), the number of samples was small and prevalence estimates may be subject to random fluctuation (**Table 4-3**).

Of 12 samples collected from clinically health quarters, nine yielded no bacterial growth and three were contaminated.

Table 4-3 Sampling results for bovine milk samples (n = 208) from quarters with clinical mastitis by participating farm and sampling period

	Farm	no growth	contaminated	gram-positive only	gram-negative only	mixed	*Prothotheca* spp.	unidentified	mild	moderate	severe	total
1st period	1	6	4	22	20	3	2	1	23	26	9	58
	2	4	3	6	14	1	0	2	7	16	7	30
	3	1	11	13	6	2	0	0	16	17	0	33
	4	1	0	2	1	1	0	0	1	2	2	5
	5	1	1	4	2	0	0	0	3	5	0	8
	6	1	4	2	5	0	0	0	3	7	2	12
	7	0	0	5	5	0	0	0	3	4	3	10
2nd period	1	5	3	6	13	2	0	0	4	17	8	29
	2	0	1	1	1	0	0	0	2	0	1	3
	3	3	3	1	7	2	0	0	6	8	2	16
	5	2	1	1	0	0	0	0	4	0	0	4
Total		24	31	63	74	11	2	3	72	102	34	208

4.3.2 Milk molecular biology – cathelicidin measurement

4.3.2.1 Protein quantification

Protein concentration results from 104 milk samples, including 95 CM samples that yield just one gram-positive or gram-negative isolate or no growth, and 9 healthy quarter milk samples that had no growth were calculated and are shown below. Protein concentration varied between 10 and 67 mg/mL. Except for other gram-negative than *E. coli* and *Klebsiella* spp. and other gram-positive than Staphylococci and Streptococci, median protein values per sample group were between 25 and 35 mg/mL. Healthy quarter milk samples showed a lower interquartile range, therefore fewer protein concentrations variations than in CM samples, and particularly less than CM that yielded *E. coli*, *Klebsiella* spp. or no growth results (**Figure 4-4**).

Bacterial growth were significantly associated with overall protein concentration (P-value = 0.031), however, for pairwise comparisons between each bacterial group at genus and species level no differences were found (P-value > 0.05). At broad category level, i.e. when differentiating bacterial growth at gram level, no growth and clinically healthy quarter, protein concentrations in the latter category were normally distributed (average (28.88

mg/mL), median (28.05 mg/mL) and in CM they were positively skewed (average higher than the median, with differences of 1.65, 1.97 and 1.82 mg/mL for CM with gram-negative, gram-positive and no growth, respectively) and had higher interquartile range (13.4, 12.95, 15.95 and 4.66 mg/mL for gram-negative, gram-positive, no growth and healthy quarter, respectively) (**Figure 4-5**). Broad category had no significant effect on overall protein concentration and in pairwise comparisons between each broad category group no differences were found. Comparing healthy quarter milk samples with CM samples of different severities, severity was significantly associated with protein concentration (P-value = 0.015). In pairwise comparisons between severity levels, protein concentration was significantly different between mild and moderate CM (P-value = 0.033). For the other pairwise comparisons, differences were not statistically significant (**Figure 4-6**).

Figure 4-4 Distribution of protein concentration in mg/mL measured through bicinchoninic acid protein assay in healthy quarter and clinical mastitis milk samples, with bacterial identification at species and genus level based on a reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Maldi-ToF MS). The black horizontal line inside each box is the median; the bottom and top of the boxes are the 1st and 3rd quartiles; vertical lines end at the minimum and maximum values; the cross is the average, the circle is an outlier.

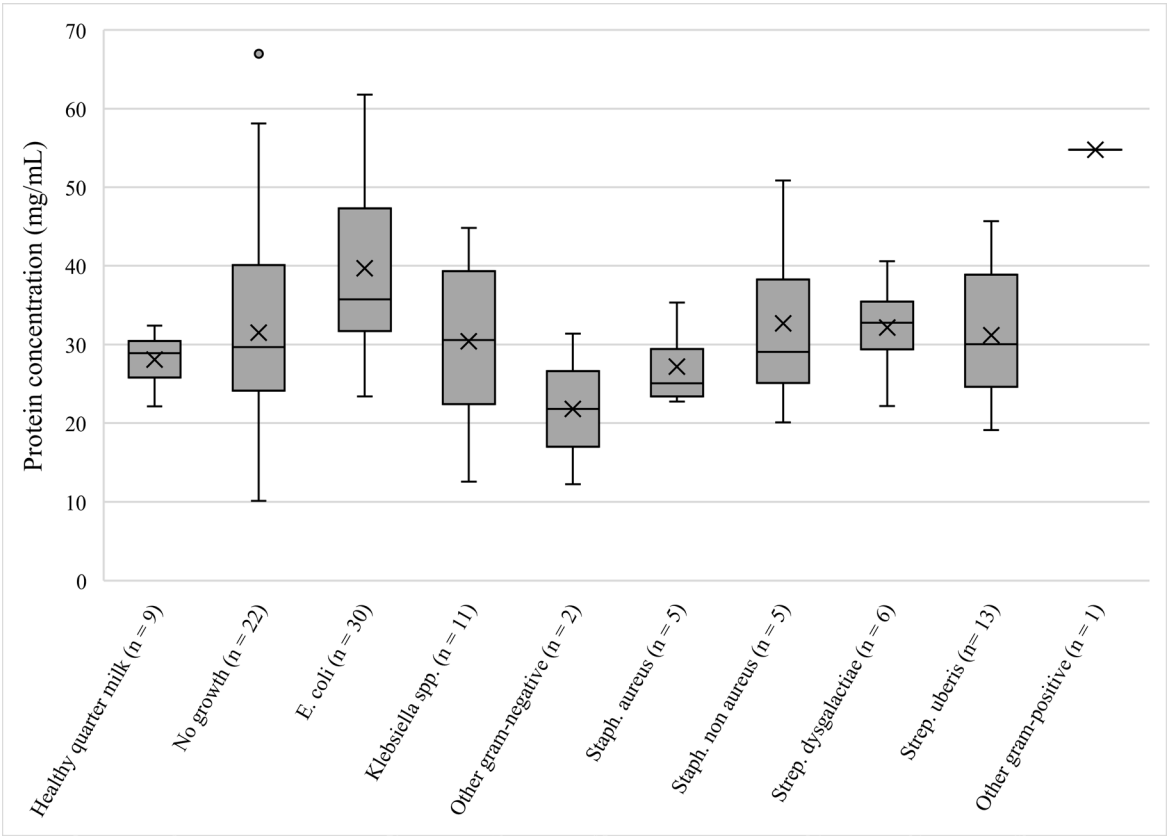


Figure 4-5 Distribution of protein concentration in mg/mL measured through bicinchoninic acid protein assay in healthy quarter and clinical mastitis milk samples The black horizontal line inside each box is the median; the bottom and top of the boxes are the first and third quartiles; vertical lines end at the minimum and maximum values; the cross is the average.

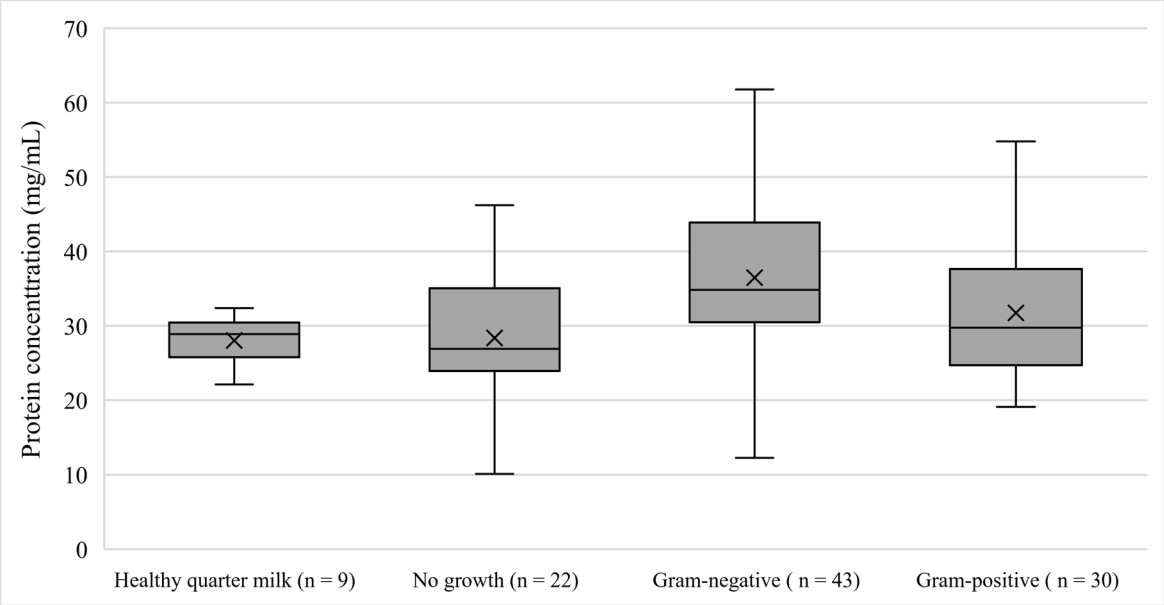
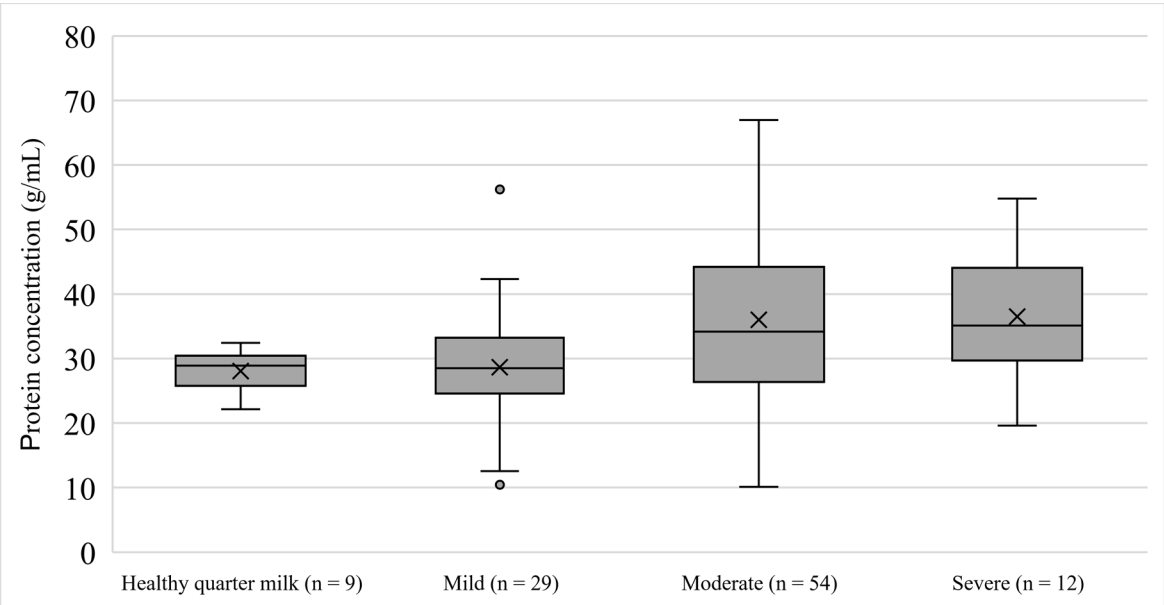


Figure 4-6 Distribution of protein concentration in mg/mL measured through bicinchoninic acid protein assay in healthy quarter and clinical mastitis milk samples with different severity levels (mild, moderate, and severe). The black horizontal line inside each box is the median; the bottom and top of the boxes are the first and third quartiles; vertical lines end at the minimum and maximum values; the cross is the average, the circle is an outlier.



4.3.2.2 Detection of cathelicidin presence in clinical mastitis milk samples

4.3.2.2.1 Western blot technique A

A subset of 60 samples was used in technique A, however, 14 samples were just run once in one gel, and therefore were excluded from the analysis. To evaluate consistency and repeatability the assay required that all the samples analysed were run twice. The remaining 46 samples included samples with no growth, and evenly balanced proportions of gram-negative and gram-positive bacteria (45.7 vs 41.3%). This subset included different gram-negative and gram-positive bacterial genera and species, and samples from different severities: mild (30.4%), moderate (58.7%) and severe (10.9%) CM (**Table 4-4**). These samples were used to evaluate the potential of the biomarker to be used as a proxy of pathogen presence.

Table 4-4 Clinical mastitis milk samples used in Western blot technique A to evaluate cathelicidin as biomarker for growth. Culture results based on the reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS).

Result	Severity			Total (%)
	Mild (%)	Moderate (%)	Severe (%)	
No growth	0 (0)	6 (13.0)	0 (0)	6 (13.0)
Growth	14 (30.4)	21 (45.7)	5 (10.9)	40 (87.0)
Gram-negative	9 (19.6)	7 (15.2)	5 (10.9)	21 (45.7)
Escherichia coli	6 (13.0)	5 (10.9)	4 (8.7)	15 (32.6)
Klebsiella spp.	3 (6.5)	1 (2.2)	1 (2.2)	5 (10.9)
Other gram-negative	0 (0)	1 (2.2)	0 (0)	1 (2.2)
Gram-positive	5 (10.9)	14 (30.4)	0 (0)	19 (41.3)
Staphylococcus spp.	1 (2.2)	4 (8.7)	0 (0)	5 (10.9)
Staphylococcus aureus	1 (2.2)	3 (6.5)	0 (0)	4 (8.7)
Non-aureus staphylococci	0 (0)	1(2.2)	0 (0)	1 (2.2)
Streptococcus spp.	4 (8.7)	10 (21.7)	0 (0)	14 (30.4)
Streptococcus dysgalactiae	2 (4.3)	6 (13.0)	0 (0)	8 (17.4)
Streptococcus uberis	2 (4.3)	4 (8.7)	0 (0)	6 (13.0)
Total	14 (30.4)	27 (58.7)	5 (10.9)	46 (100)

When using the technique, A, the biomarker had low to moderate sensitivity for growth (range 22.0-55.0%). Specificity was higher than sensitivity, but with a more marked variation of values between gels (A1 and A2) and both exposures (range 33.3-100%). Accuracy was poor for this outcome (< 54.3%). Positive predictive values were moderate to high (> 69.2%) but NPV were low (< 18.8%), explained by the high prevalence of bacterial growth (87%) in the sample set used. The ability of cathelicidin as a biomarker to detect gram-positive bacteria was better than for detection of growth, showing moderate sensitivity (range 31.26-68.4%) and specificity (range 55.6-81.55%), translating into moderate accuracy for gram-positive organisms (>56.5%) (**Table 4-5**).

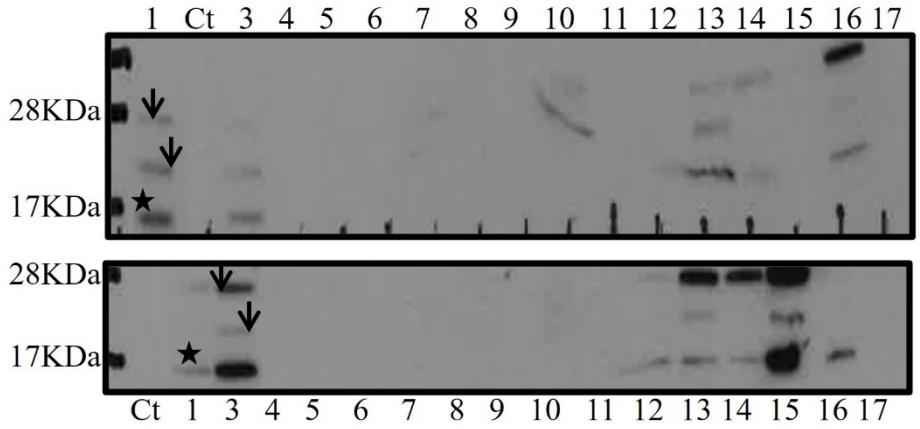
When assessing different exposure times in different gels, sensitivity and specificity were affected differently, which is a common trade-off, i.e. when the sensitivity increased the specificity decreased for increased exposure times. The different gels showed inconsistencies in the results with the same exposure and running the same samples. For the samples from non-severe CM cases (n = 41), the potential of cathelicidin to be used as a treatment decision support tool was evaluated. Sensitivity for the subset of non-severe CM cases was similar to Se for all CM cases (47.7 vs 47.4%), but specificity was slight decreased (68.2 vs 74.1%). Predictive values for gram-positive results and treatment were low to moderate (< 71.4%) for both outcomes (**Table 4-5**).

Table 4-5 Performance of the culture-independent biomarker under evaluation, cathelicidin, using western blot technique A for detection of mastitis pathogens (n = 46 samples), using different exposure times (30 seconds and 5 minutes), and running the gels twice (A1 and A2). Results are based on comparison with a reference test consisting of culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Values represent point estimates expressed as percentage with 95% confidence intervals in brackets.

	30 seconds exposure		5 minutes exposure	
	A1	A2	A1	A2
Sensitivity				
Growth	35.0 [21.2; 48.8]	22.5 [10.4; 34.6]	55.0 [40.6; 69.4]	37.5 [23.5; 51.5]
Gram-positive	47.4 [32.9; 61.8]	31.6 [18.1; 45.0]	68.4 [55.0; 81.9]	52.6 [38.2; 67.1]
Treatment	47.7 [32.1; 62.7]	31.6 [17.4; 45.8]	68.4 [54.2; 82.6]	52.6 [37.3; 67.9]
Specificity				
Growth	100 [100-100]	33.3 [19.7; 47.0]	50.0 [35.6; 64.4]	33.3 [19.7; 47.0]
Gram-positive	81.5 [70.3; 92.7]	74.1 [61.4; 86.7]	55.6 [41.2; 69.9]	66.7 [53.0; 80.3]
Treatment	81.8 [70.0; 93.6]	68.2 [53.9; 82.4]	50.0 [34.7; 65.3]	59.1 [44.0; 74.1]
Accuracy				
Growth	43.5 [29.2; 57.8]	23.9 [11.6; 36.2]	54.3 [40.0; 68.7]	37.0 [23.0; 50.9]
Gram-positive	67.4 [53.8; 80.9]	56.5 [42.2; 70.8]	60.9 [46.8; 75.0]	60.9 [46.8; 75.0]
Treatment	65.9 [51.3; 80.4]	51.2 [35.9; 66.5]	58.5 [43.5; 73.6]	56.1 [40.9; 71.3]
Positive predictive value				
Growth	100 [100-100]	69.2 [55.9; 82.6]	88.0 [78.6; 97.4]	78.9 [67.2; 90.7]
Gram-positive	64.3 [50.4; 78.1]	46.2 [31.7; 60.6]	52.0 [37.6; 66.4]	52.6 [38.2; 67.1]
Treatment	69.2 [55.1; 83.4]	46.2 [30.9; 61.4]	54.2 [38.9; 69.4]	52.6 [37.3; 67.9]
Negative predictive value				
Growth	18.8 [7.5; 30.0]	6.1 [0; 13.0]	14.3 [4.2; 24.4]	7.4 [0; 15.0]
Gram-positive	68.8 [55.4; 82.1]	60.6 [46.5; 74.7]	71.4 [58.4; 84.5]	66.7 [53.0; 80.3]
Treatment	64.3 [49.6; 79.0]	53.6 [38.3; 68.6]	64.7 [50.1; 79.3]	59.1 [44.0; 74.1]

Repeatability of the technique, measured as the agreement between gels using technique A, and expressed in Cohen's κ, was slight for exposure of 30 seconds ($\kappa = 0.109$) and moderate for exposure of 5 minutes ($\kappa = 0.572$). An example of inconsistencies is shown in **Figure 4-7**.

Figure 4-7 Western blot technique A with 5 min exposure demonstrating the appearance of the cathelicidin forming multiple bands, a monomer seen in the low molecular weight band (17 kDa, examples illustrated by the stars), and potential multiple dimer bands seen for the other molecular weight bands (> 18 kDa examples illustrated by the arrows) (gels A1 and A2). This figure shows inconsistency between gels. Ct: negative control. Consistent results 1-9, 11, 13-14 and 16-17. Inconsistent results: 10, 12 and 15. Interpreted as samples positive in both gels 1, 3, 12-14 and 16. Samples interpreted as negative in both gels: 4-9 and 15, 17.



4.3.2.2.2 Western blot technique B

The improved technique was achieved after several trial-and-error assays, when consistencies in the signal, bands size, absence of outliers and minimum non-specific background were achieved after several technical repeats, including multiple lanes with the same sample in a single gel, and multiple gels with the same samples. The optimum conditions for gel loading included 10 µL of the solution with a protein concentration at 2 mg/mL, 4 µL of protein molecular weight ladder in the first well of the gel, and positive and negative controls in second and third wells, respectively. Ideal sample incubation conditions included heating at 95°C for 4 min. Sample buffer with standard β-mercaptoethanol concentration as in technique A resulted in presence of cathelicidin monomer and dimer (Gels B3 and B4) (**Figure 4-9**) and sample buffer with an increase in β-mercaptoethanol concentration of 50% resulted in disruption of the dimer formation (Gels B1 and B2) (**Figure 4-8**). Both concentrations were used to evaluate the potential of the biomarker as a proxy of pathogen presence.

A subset of 35 samples was used in technique B, including samples with no growth (14.7%), a higher proportion of gram-negative than gram-positive samples (61.8 vs 23.5%),

and samples from different severities: mild (23.5%), moderate (58.8) and severe (17.6%) (**Table 4-6**).

Table 4-6 Clinical mastitis milk samples used in Western blot technique B results based on the reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS)

Result	Severity			Total (%)
	Mild (%)	Moderate (%)	Severe (%)	
No growth	1 (2.9)	4 (11.8)	0 (0)	5 (14.7)
Growth	7 (20.6)	16 (47.1)	6 (17.6)	29 (85.3)
Gram-negative	4 (11.8)	11 (32.4)	6 (17.6)	21 (61.8)
Escherichia coli	4 (11.8)	9 (26.5)	5 (14.7)	18 (52.9)
Klebsiella spp.	0 (0)	1 (2.9)	1 (2.9)	2 (5.9)
Other gram-negative	0 (0)	1 (2.9)	0 (0)	1 (2.9)
Gram-positive	3 (8.8)	5 (14.7)	0 (0)	8 (23.5)
Staphylococcus spp.	1 (2.9)	0 (0)	0 (0)	1 (2.9)
Staphylococcus aureus	1 (2.9)	0 (0)	0 (0)	1 (2.9)
Non-aureus staphylococci	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus spp.	2 (5.9)	5 (14.7)	0 (0)	7 (20.6)
Streptococcus dysgalactiae	1 (2.9)	3 (8.8)	0 (0)	4 (11.8)
Streptococcus uberis	1 (2.9)	2 (5.9)	0 (0)	3 (8.8)
Total	8 (23.5)	20 (58.8)	6 (17.6)	34 (100)

Sample buffer that disrupts cathelicidin dimer in the gels (B1 and B2):

When using the higher β-mercaptoethanol concentration for the sample buffer (gels B1 and B2) there was a total or partial suppression of the higher molecular weight cathelicidin bands, disrupting cathelicidin dimer formation limiting cathelicidin appearance in a gel to a single monomer band with lower molecular weight (17 kDa) (**Figure 4-8**). In technique B using an increase in the β-mercaptoethanol concentration of 50%, the biomarker had moderate to high sensitivity to detect bacterial growth (sensitivity range 62.1-79.3%), however, specificity for growth was inferior to sensitivity (range 40.0-80.0%), which was reflected in moderate accuracy (range 61.8-73.5%) (**Table 4-7**). Positive predictive values were high (>88.5%) in contrast to negative predictive value (< 30.8%), explained by the high prevalence of bacterial growth (85.3%) in the sample set used. Regarding the ability of the biomarker to detect gram-positive bacteria using this technique and the mentioned reducing agent concentration in the sample buffer, the biomarker performance was lower than for detection of growth, showing low to moderate sensitivity (range 37.5-75.0%) and even lower specificity (range 23.1-38.5%) (**Table 4-7**).

As for technique A, when assessing different exposure times in different gels, sensitivity increased, and specificity decreased for increased exposure times. Using this protocol, the agreement between gels was better than for technique A, with a substantial and perfect agreement for exposure of 30 seconds (κ = 0.693) and 5 minutes (κ = 1.0), respectively.

For the samples from non-severe CM cases (n = 28), the potential of cathelicidin to be used as a treatment decision support tool was evaluated. Sensitivity for gram-positive in the subset of non-severe CM cases had the same results as when severe CM cases were included in this sample set. Specificity improved but was still poor (< 45.0%). Predictive values had a wide range for growth, gram-positive and treatment, ranging from 15-95.2% (**Table 4-7**).

Figure 4-8 Western blot technique B with 5 min exposure, demonstrating appearance of a single cathelicidin band monomer with low molecular weight (17kDa) (gels B1 and B2) and example of consistency in cathelicidin detection. 1: Protein molecular weight ladder, Ct+: positive control and Ct-: negative control, consistent results for all the tested samples 4-15. Interpreted as samples positive in both gels 4, 6-11, 13 and 15. Samples negative: 5, 12 and 14.

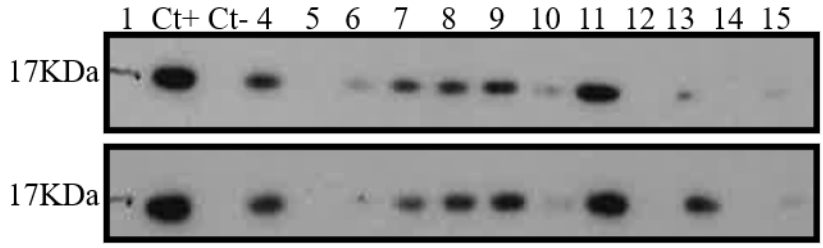


Table 4-7 Performance of the culture-independent biomarker under evaluation, cathelicidin, using western blot technique B for detection of mastitis pathogens (n = 35 samples), using different exposure times (30 seconds and 5 minutes), and running the gels twice using a sample buffer that disrupts cathelicidin dimer (B1 and B2). Results are based on comparison with a reference test consisting of culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Values represent point estimates expressed as percentage with 95% confidence intervals in brackets.

	30 seconds exposure		5 minutes exposure	
	B1	B2	B1	B2
Sensitivity				
Growth	69.0 [53.4; 84.5]	62.1 [45.8; 78.4]	79.3 [65.7; 92.9]	79.3 [65.7; 92.9]
Gram-positive	62.5 [46.2; 78.8]	37.5 [21.2; 53.8]	75.0 [60.4; 89.6]	75.0 [60.4; 89.6]
Treatment	62.5 [44.6; 80.4]	37.5 [19.6; 55.4]	75.0 [59.0; 91.0]	75.0 [59.0; 91.0]
Specificity				
Growth	80.0 [66.6; 93.4]	60.0 [43.5; 76.5]	40.0 [23.5; 56.5]	40.0 [23.5; 56.5]
Gram-positive	38.5 [22.1; 54.8]	34.6 [18.6; 50.6]	23.1 [8.9; 37.2]	23.1 [8.9; 37.2]
Treatment	45.0 [26.6; 63.4]	40.0 [21.9; 58.1]	30.0 [13.0; 47.0]	30.0 [13.0; 47.0]
Accuracy				
Growth	70.6 [55.3; 85.9]	61.8 [45.4; 78.1]	73.5 [58.7; 88.4]	73.5 [58.7; 88.4]
Gram-positive	44.1 [27.4; 60.8]	35.3 [19.2; 51.4]	35.3 [19.2; 51.4]	35.3 [19.2; 51.4]
Treatment	50.0 [31.5; 68.5]	39.3 [21.2; 57.4]	42.9 [24.5; 61.2]	42.9 [24.5; 61.2]
Positive predictive value				
Growth	95.2 [88.1; 100]	90.0 [79.9; 100]	88.5 [77.7; 99.2]	88.5 [77.7; 99.2]
Gram-positive	23.8 [9.5; 38.1]	15.0 [3.0; 27.0]	23.1 [8.9; 37.2]	23.1 [8.9; 37.2]
Treatment	31.3 [14.1; 48.4]	20.0 [5.2; 34.8]	30.0 [13.0; 47.0]	30.0 [13.0; 47.0]
Negative predictive value				
Growth	30.8 [15.3; 46.3]	21.4 [7.6; 35.2]	25.0 [10.4; 39.6]	25.0 [10.4; 39.6]
Gram-positive	76.9 [62.8; 91.1]	64.3 [48.2; 80.4]	75.0 [60.4; 89.6]	75.0 [60.4; 89.6]
Treatment	75.0 [59.0; 91.0]	61.5 [43.5; 79.6]	75.0 [59.0; 91.0]	75.0 [59.0; 91.0]

Sample buffer that shows cathelicidin dimer in the gels (B3 and B4):

When using the lower β-mercaptoethanol concentrations (gels B3 and B4) it showed two cathelicidin bands between 17-28 kDa (**Figure 4-9**). Using this sample buffer, the sensitivity of the biomarker to detect growth increased compared to the sample buffer used in the gels presented before (B1 and B2) (range 75.9-89.7% vs 62.1-79.3%), but the specificity decreased (range 40.0-60.0% vs 62.1-79.3%), resulting in moderate to high accuracy for growth (range 73.5-82.4%), which was better than for gels B1 and B2. For detection of gram-positive bacteria, sensitivity was much higher than specificity (range 75.0-87.5% vs 15.4-30.8%), resulting in poor accuracy (range 29.4-41.2%) (**Table 4-8**).

As before, sensitivity increased, and specificity decreased for increased exposure times. The agreement between gels B3 and B4 as expressed in Cohen's κ revealed the repeatability of the technique was almost perfect for exposure of 30 seconds (κ = 0.9) and substantial for exposure of 5 minutes (κ = 0.7), which was higher than repeatability in technique A which was slight (κ = 0.109) and moderate for (κ = 0.572) for 30 seconds and 5 minutes exposures, and similar to the agreement between gels B1 and B2, which had a substantial and perfect agreement for exposure of 30 seconds (κ = 0.693) and 5 minutes (κ = 1.0), respectively.

Sensitivity for gram-positive in the subset of non-severe CM cases (n = 28) was the same as for severe CM cases in this sample set. Specificity improved but was still poor (< 50.0%).

Figure 4-9 Western blot technique B with 5 min exposure, demonstrating the appearance of the cathelicidin forming two bands, a monomer seen in the low molecular weight band (17 kDa shown by the star), and a dimer seen in the high molecular weight band (28 kDa shown by the arrow) (gels B3 and B4) and showing consistency in cathelicidin detection. Ct+: positive control and Ct-: negative control. Consistent results 4-15. Interpreted as samples positive in both gels 4-13 and 15. Samples negative in both gels: 3-11 and 17.

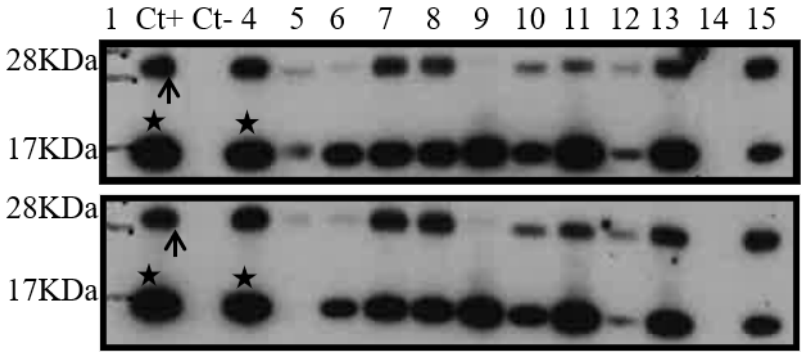


Table 4-8 Performance of the culture-independent biomarker under evaluation, cathelicidin, using western blot technique B for detection of mastitis pathogens (n = 35 samples), using different exposure times (30 seconds and 5 minutes), and running the gels twice using a sample buffer that does not interfere with cathelicidin dimer (B3 and B4). Results are based on a comparison with a reference test consisting of culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Values represent point estimates expressed as percentage with 95% confidence intervals in brackets.

	30 seconds exposure		5 minutes exposure	
	B3	B4	B3	B4
Sensitivity				
Growth	75.9 [61.5; 90.2]	79.3 [65.7; 92.9]	86.2 [74.6; 97.8]	89.7 [79.4; 99.9]
Gram-positive	75.0 [60.4; 89.6]	75.0 [60.4; 89.6]	75.0 [60.4; 89.6]	87.5 [76.4; 98.6]
Treatment	75.0 [60.4; 89.6]	75.0 [59.0; 91.0]	75.0 [59.0; 91.0]	87.5 [75.3; 99.8]
Specificity				
Growth	60.0 [43.5; 76.5]	60.0 [43.5; 76.5]	40.0 [23.5; 56.5]	40.0 [23.5; 56.5]
Gram-positive	30.8 [15.3; 46.3]	26.9 [12.0; 41.8]	15.4 [3.3; 27.5]	15.4 [3.3; 27.5]
Treatment	50.0 [33.2; 66.8]	30.0 [13.0; 47.0]	20.0 [5.2; 34.8]	20.0 [5.2; 34.8]
Accuracy				
Growth	73.5 [58.7; 88.4]	76.5 [62.2; 90.7]	79.4 [65.8; 93.0]	82.4 [69.5; 95.2]
Gram-positive	41.2 [24.6; 57.7]	38.2 [21.9; 54.6]	29.4 [14.1; 44.7]	32.4 [16.6; 48.1]
Treatment	55.9 [39.2; 72.6]	42.9 [24.5; 61.2]	35.7 [18.0; 53.5]	39.3 [21.2; 57.4]
Positive predictive value				
Growth	91.7 [82.4; 100]	92.0 [82.9; 100]	89.3 [78.9; 99.7]	89.7 [79.4, 99.9]
Gram-positive	25.0 [10.4; 39.6]	24.0 [9.6; 38.4]	21.4 [7.6; 35.2]	24.1 [9.8; 38.5]
Treatment	31.6 [16.0; 47.2]	30.0 [13.0; 47.0]	27.3 [10.8; 43.8]	30.4 [13.4; 47.5]
Negative predictive value				
Growth	30.0 [14.6; 45.4]	33.3 [17.5; 49.2]	33.3 [17.5; 49.2]	40.0 [23.5; 56.5]
Gram-positive	80.0 [66.6; 93.4]	77.8 [63.8; 91.8]	66.7 [50.8; 82.5]	80.0 [66.6; 93.4]
Treatment	86.7 [75.2; 98.1]	75.0 [59.0; 91.0]	66.7 [49.2; 84.1]	80.0 [65.2; 94.8]

4.3.2.2.3 Cathelicidin results by pathogen and severity

From the gels and exposures that yield perfect agreement (B1 and B2, 5 minutes exposure) and almost perfect agreement (B3 and B4, 30 seconds exposure) cathelicidin results were analysed by genus and species level, severity level and broad categories when differentiating bacterial growth at gram level and no growth. Results in each pair of tests were similar, except for one sample in B3 and B4 gels with 30 seconds exposure (corresponding to lane 5 in **Figure 4-8** and **Figure 4-9**), which was a mild case of *E. coli*. From a total of 34 samples, cathelicidin was detected in most of the samples (76.1%). It was detected in samples that had no growth and growth, and in a similar proportion of gram-positive and gram-negative samples (**Figure 4-10**). At species and genus level, cathelicidin was not detected in the only *Staph. aureus* and only "other gram-negative" samples, which was *Pasteurella multocida*. For the remainder of samples with growth, cathelicidin was detected in more than half of the samples of *E. coli* and *Strep. dysgalactiae* and in all the samples that yielded *Klebsiella* spp. and *S. uberis* (**Figure 4-10**). In the tested sample set the proportion of samples where cathelicidin was detected increased with severity (**Figure 4-11**).

Figure 4-10 Cathelicidin detection using western blot technique B, gels B1 and B2 with 5 minutes exposure, using clinical mastitis samples results where there was perfect agreement between gels (n = 34). Bacterial identification at species and genus levels based on a reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS).

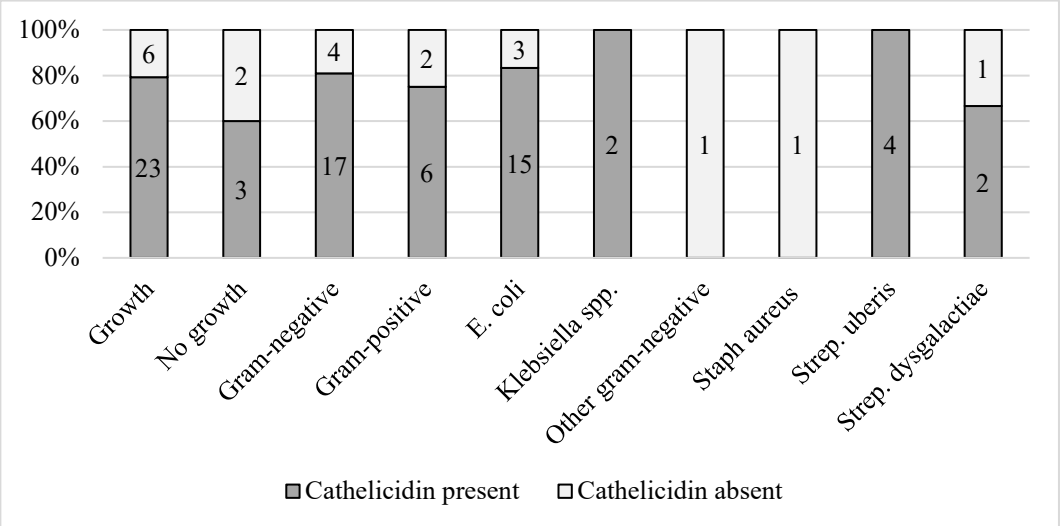
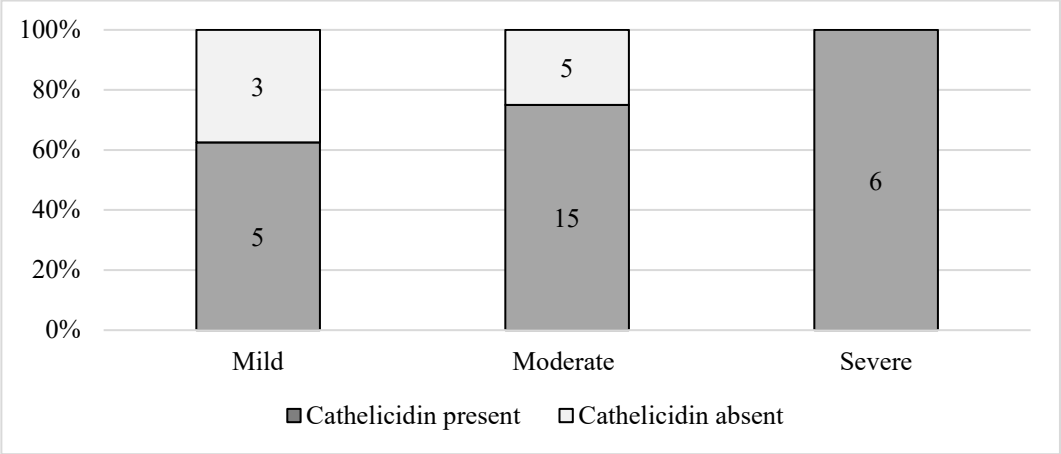


Figure 4-11 Cathelicidin detection using western blot technique B, gels B1 and B2 with 5 minutes exposure, using clinical mastitis samples with different severity levels (mild, moderate, and severe) where there was perfect agreement between gels (n = 34).



4.3.3 Agreement between techniques A and B

The agreement between Techniques A and B on the same samples (n = 18) as expressed in Cohen's κ, revealed poor reproducibility of the biomarker with κ values varying from slight, fair and moderate for one comparison (A1 and B2 for 5 minutes exposure) (**Table 4-9**).

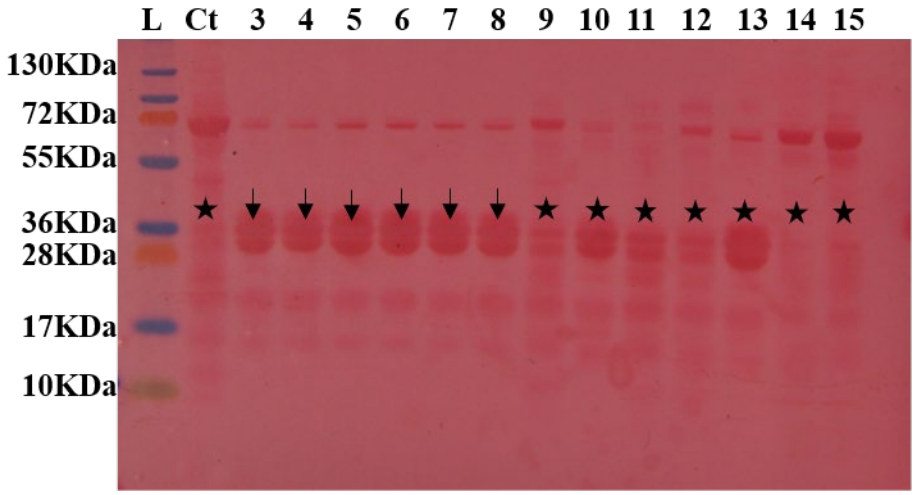
Table 4-9 Cohen's κ agreement between gels of different technique A and B using the same samples (n = 18) and two different exposures of 30 seconds and 5 minutes. The values represent κ Cohen's agreement. NA: not available.

Exposure	Technique and gels	B1	B2	B3	B4
30 seconds	A1	0.40	0.36	0.25	0.25
	A2	0.14	0.12	0.16	0.16
5 minutes	A1	0.36	0.48	0.17	NA
	A2	0.11	0.11	-0.11	NA

4.3.4 Milk protein profiles

The healthy quarter milk samples had similar milk protein profiles (samples with numbers 3-8 **Figure 4-12**) and generally differed from milk protein profiles of CM (Ct and 9-15 **Figure 4-12**). In healthy quarter milk samples, the dominant proteins are caseins (represented by the arrows in 28-40 kDa). In CM samples (CT and 9-15), there is an alteration in casein profile, whereby bands often seemed of smaller size and there is an increase of other proteins of higher molecular weight (72-80 kDa) (**Figure 4-12**).

Figure 4-12 Ponceau S staining of the blot, and protein profiles of different clinically healthy quarter milk samples (3-8) and clinical mastitis samples with no growth (Ct, 9-15). L: protein molecular weight ladder, Ct: control. Arrows indicate casein apparent similar profile. Stars indicate casein altered profile.



4.4 Discussion

In the present study, I evaluated if cathelicidin could be used to differentiate gram-positive CM from other causes of mastitis and if this biomarker could be used to inform treatment decisions in non-severe CM. Cathelicidin in the present study was detected in CM milk samples using two different western blot techniques adapted from previous studies (Smolenski *et al.*, 2011; Shafie *et al.*, 2013).

A good biomarker must exhibit good sensitivity, specificity, accuracy, be repeatable and reproducible (Boehmer, 2011). Cathelicidin in the present study showed to be a poor biomarker to inform treatment decisions, because it had limited capacity to differentiate gram-positive CM from other causes of CM, regardless of methodology. To detect gram-positive bacteria technique A showed variable sensitivity (< 68.4%) and specificity (< 81.5%), with moderate accuracies for different gels and exposures (< 67.4%), which is not sufficient for diagnostic use. In addition, repeatability was considered an issue using this technique, probably due to extra and more skilled steps needed when using the transfer in a blotting tank than when the transfer used a semi-dry blotting chamber. The fact that both techniques A and B were not performed simultaneously also can explain why technique A performed poorly, as it was done first, and it requires time to acquire consistency in each step. There may be a learning effect for the person conducting the experiments. Technique B was performed to overcome repeatability issues, which was demonstrated by having almost perfect and perfect agreement between two gels using the same samples in two different exposures. In these conditions, it was possible to evaluate biomarker performance, showing moderate-high sensitivity (75%) but poor specificity (23.1%) for detecting gram-positive CM. When excluding severe cases, specificity increased slightly (30%), however, not enough to be considered an accurate test (35% accuracy) and capable to inform treatment decisions. For use in an automatic milking system, sensitivity and specificity requirements are 80% and 99%, respectively (ISO, 2017). It can be argued that techniques were applied to distinct sample sets, with different bacterial prevalence and different sample sizes, however, sensitivity, specificity and accuracy, in contrast to predictive values, are not influenced by bacterial prevalence (Dohoo *et al.*, 2009). The low sample size in this study decreases its power and explains the wide confidence intervals around the point of estimate. With larger numbers of samples, the confidence intervals could be narrowed, and differences

might become significant, but there would still be too many false-positive and false-negative results to make this an accurate diagnostic test.

Other studies that investigated the abundance of the biomarker at the genus and species level using ELISA techniques also demonstrated that the biomarker has limited discriminatory power to differentiate CM caused by different bacteria at the genus and species level (Addis *et al.*, 2017; Wollowski *et al.*, 2021). This supports the results in the present study, where specificity was considered the main issue. In one study cathelicidin abundance could discriminate only between *Strep. agalactiae* and coagulase negative staphylococci, which demonstrated the maximum and the minimum levels of cathelicidin, respectively (Addis *et al.*, 2017). In another study, cathelicidin levels in CM were not influenced by the mastitis-causing pathogen, and the significant differences detected for cathelicidin levels were limited to SCM cases caused by *S. uberis*, *Staph. aureus* and *Staph non-aureus* (Wollowski *et al.*, 2021). These previous studies used quantitative methods to quantify cathelicidin concentrations in each CM sample, which have been reported to be more sensitive than western blot techniques (Addis *et al.*, 2017). Cathelicidin in the present study was not quantified, as repeatability was identified as an issue even when cathelicidin detection was just considered. The disruption of cathelicidin dimers was achieved in my study by increasing reducing agent concentration in the sample buffer, as previously observed for cathelicidin dimer (Storici *et al.*, 1996) and for transthyretin protein in CSF samples (Shafie *et al.*, 2013). This was done in the present study in an attempt to quantify both cathelicidin forms, but quantification was not done because of the poor repeatability of results, even for qualitative reading. The use of a numerical reading would have allowed the determination of a cut-off to optimise both sensitivity and specificity or to maximise one of them (Watson and Petrie, 2010).

Reproducibility of the assay is also an important characteristic when evaluating a biomarker. Reproducibility can be assessed when there is satisfactory repeatability of a procedure and assesses whether two different individuals using the same methodology obtain similar results or whether two different techniques performed under similar circumstances produce the same results, evaluating thus between-method or between-observer agreement (Watson and Petrie, 2010). In the present study, the between method agreement was assessed (technique A vs technique B) when aliquots of the same samples were used in both techniques. However, for logical reasons due to poor repeatability of

technique A reproducibility of the biomarker in the conditions evaluated was low but reproducibility assessment under these circumstances is unreliable (Watson and Petrie, 2010). In the current study, assessment of reproducibility using the between-observer agreement, i.e. technique B performed by two different people in the same samples, would have been a reliable way to assess it. Due to time constraints, this was not performed.

Because of time restrictions caused by Covid lockdowns, the sample set used for technique B was smaller than in technique A, with uneven distributions of gram-positive CM samples. The uneven prevalence's used explains the differences in positive and negative predictive values. As seen in **Chapter 3**, predictive values regarding treatment decision are more important for on-farm application of the biomarker in a specific population than other point estimates, as they inform about targeted treatment decisions performance. A high positive predictive value is associated with an optimal reduction of AMU and a high negative predictive value means that treatment is only withheld when cows truly do not need it (Malcata *et al.*, 2020). Unfortunately, these interpretations cannot be done in the present study. Other limitation of the sample set used, was that it did not allow investigation of the potential of the biomarker to differentiate major and minor mastitis pathogens. This could have been of relevance for implementing targeted treatment decisions, as CM caused by NAS generally do not require antimicrobial treatment (McDougall, 1998; Wilson *et al.*, 1999).

The biomarker showed limited specificity to detect gram-positive CM. High specificity for this outcome would have meant that cathelicidin would not be detected in gram-negative cases and culture-negative cases. This low specificity is not an unexpected result as the biomarker has been used as indicator of mastitis in general rather (Smolenski *et al.*, 2014; Pongthaisong *et al.*, 2015; Addis *et al.*, 2016). The present study supported this notion, as none of the healthy quarter milk samples tested were positive for the presence of cathelicidin, demonstrating 100% specificity of this peptide as a mastitis biomarker. These results agree with previous studies where cathelicidin in healthy quarters was not detected (Smolenski *et al.*, 2011; Addis *et al.*, 2017), and supports the knowledge that there is a need for udder tissue inflammation for the biomarker to be released in milk (Cubeddu *et al.*, 2017). This can be caused by IMI, which are generally detectable in culture, but is not infrequent that bacteriological culture yields no growth results, e.g.13.6% in the present study, which is within reported ranges from 10-40% (Roberson, 2003). Culture-negative results are

intrinsic characteristics of bacterial culture that also explain in part the poor specificity of the biomarker for gram-positive bacteria, as a false negative result in the reference test would erroneously explain a false positive result for the biomarker (Addis *et al.*, 2016).

A strategy to increase the specificity of this biomarker as an indicator to inform targeted CM treatment would be to use monoclonal antibodies for several specific subtypes of cathelicidin (Young-Speirs *et al.*, 2018) rather than a polyclonal antibody that has affinity to all subtypes of cathelicidins This may be possible by using antibodies specific to cathelicidins -5, -6 and -7 as they have been detected in naturally occurring gram-positive mastitis (Ibeagha-Awemu *et al.*, 2010; Huang *et al.*, 2014) or experimentally induced IMI with this type of bacteria (Reinhardt *et al.*, 2013; Mudaliar *et al.*, 2016). Cathelicidins-5 and -7 have are increased in *S. uberis* infections (Mudaliar *et al.*, 2016) and cathelicidin-6 and -7 are increased in *Staph. aureus* infections (Reinhardt *et al.*, 2013; Huang *et al.*, 2014). By contrast, mastitis caused by *E. coli* or lipopolysaccharide challenge have increased cathelicidin-1, -2, -3 and -4 (Boehmer *et al.*, 2008; Danielsen *et al.*, 2010; Ibeagha-Awemu *et al.*, 2010; Yang *et al.*, 2015), but none of these studies reported an increase in cathelicidin -5, -6 and -7. However, these results are often reported in experimental challenge studies. Therefore, there is a need to investigate if similar effects occur in naturally occurring CM caused by the same MCB or other gram-positive and gram-negative bacteria that have not been investigated yet. Differences between challenge infections and natural occurring CM studies in *Staph. aureus*, report differences in types of cathelicidins detected, with cathelicidin-2, -6, -3, -4 in one study (Huang *et al.*, 2014) and cathelicidin-1, -2, -4, -6 and -7 in the other (Reinhardt *et al.*, 2013). Different studies also reported differences in the fold increase for the same cathelicidin, for example from 1.4 fold (Huang *et al.*, 2014) to -20-fold (Reinhardt *et al.*, 2013) for cathelicidin-6.

The present method detected cathelicidin in most of the CM samples tested (26/34, 76.47%), which demonstrate that different CM cases yield different cathelicidin responses, despite the presence or absence of bacterial growth in bacteriological results all these samples were from cases of CM. Cathelicidin levels may have been influenced by the bacterial numbers at the time of sampling. In an experimentally induced IMI with *S. uberis*, using proteomic analysis at multiple time points pre- and post-challenge, the cathelicidin peak coincided with the highest decrease of bacterial numbers (Mudaliar *et al.*, 2016). In the present study, increased severity was associated with numerically higher proportions of CM

where cathelicidin was detected. The only other study that evaluated this association showed no effect (Wollowski *et al.*, 2021). Host factors may contribute to the variation in cathelicidin, as it was shown in an experimental challenge with the lipopolysaccharides that cathelicidin results varied among the three challenged animals (Danielsen *et al.*, 2010). Bacterial strain effects may also contribute to cathelicidin levels, as hypothesized in a previous study in other biomarkers (Thomas *et al.*, 2018).

Other relevant outcomes of this study were that both bacterial presence and severity were significantly associated with increased protein concentration in milk. This is not an incidental finding but supports the purpose of this research in how severity and bacteria can affect cathelicidin. In this study, protein expression differed between CM and healthy quarter milk samples, which was shown by significant differences in protein concentrations in mild and moderate CM, and differences in CM milk profile detected through electrophoresis. Whey from healthy quarter milk samples is characterized by the abundance of casein (80% of total protein) and whey proteins (10-37 kDa) which represent about 16% of total milk protein. In CM samples protein profile is characterized by the increased abundance of serum albumin and other vascular-derived proteins such as serum albumin, serotransferrin, fibrinogen, etc., due to break down of blood milk barrier (60-100 kDa) (Boehmer *et al.*, 2008, 2010). These proteins that leak in the milk from systemic circulation are large glycoproteins. As reported by Mudaliar *et al.* (2016), concentrations of some of these proteins can be increased or decreased during the process of inflammation and at different periods after challenge. Increase of some of these proteins can be more than 25000-fold, others can decrease more than 5000-fold. In the present study, CM samples were collected when farm-staff detected the CM, which will have occurred at different stages of infection. This can potentially explain the high variability of protein concentrations presented for the CM cases. The discrepancy of protein concentrations being due to the preparation method seems unlikely as the median and average protein concentration in healthy quarter milk samples (28.88 and 28.05 mg/mL, respectively) were comparable to the reported average milk protein concentration in normal milk (32 mg/mL) (Mudaliar *et al.*, 2016).

Other important point of discussion is that the proportion of culture-negative samples in the present study was 13.6%, which was within the reported ranges but probably on the lower side. For example, in a previous study in the UK, the proportion of culture-negative results among samples from CM was 14% (Milne et al., 2002) which is similar to the one

found in the present study. However, studies from North America reported prevalence's from 10-40% (Roberson, 2003). The thawing step of up to 8 hours as discussed in Chapter 3 might have had an impact in my results acting as a pre-incubation step. The IMI definition used (100 colony-forming unit/mL) is other factor that may have influenced the proportion of no growth samples as a more lenient or strict IMI definitions would have had yielded different results (Dohoo *et al.*, 2011). Prudence is required when comparing present prevalence results to other studies as bacteriology techniques and IMI definitions may also differ among them.

Chapter 5. General discussion

Knowledge development has always been a synonym for opportunity and change. Knowledge regarding technological development, demonstrated by examples of sophisticated computers, mobile phones, almost ubiquitous available network, or by recent DNA-sequencing technologies, allowed impressive changes in our lives and in our perception of the world. The biology of CM, however, did not change as much and certainly not at the same speed. Regarding udder health, there have been improvements and changes in genetics and husbandry, and new ways to harvest milk. Some of these changes were subsequently responsible for important shifts in mastitis pathogens' prevalence in some countries (Zadoks and Fitzpatrick, 2008).

Societal concerns regarding AMU have also changed and they influence the availability and AMU for CM treatment. However, more reluctant to change is human behaviour, which drives the need, the development and uptake of diagnostic tools and implementation of targeted selective treatment. Whilst the previous two chapters have focussed on technical or scientific aspects of new point-of-care diagnostics, social or convenience aspects of diagnostic tests are equally as important to their use. In this section, I discuss farmers' behaviour and mindset and the drivers and barriers for behavioural change towards targeted treatment decisions and uptake of on-farm diagnostics.

5.1 Drivers of diagnostic test uptake

5.1.1 Current practices – farmers' behaviour

As seen in previous chapters, knowledge to support CM targeted treatment decisions is available. However, it is well recognised from previous udder health studies that farmers infrequently implement them, even though they know that they would benefit from the adoption of new management practices (Barkema, *et al.*, 1999), and their udder health management decisions are sometimes not fully understood (Vaarst *et al.*, 2002). Farmer behaviour can explain, to some extent, differences in the occurrence of mastitis problems between farms (Jansen *et al.*, 2009). Clinical mastitis treatment approaches differ in different countries and farms, and overall, it seems that there is a big opportunity to improve current

CM practices and promote prudent AMU. In France, it is still very common to treat all cases of CM with antimicrobials (Samson *et al.*, 2016). In the Netherlands, just 50% of the farmers reported using blanket treatment of all CM (Griffioen *et al.*, 2016). In Wales and England, CM with "watery milk" or "clots" was described as the health condition where the farmers are most likely to use antimicrobial (Jones *et al.*, 2015). Despite these differences, it seems broadly accepted that farmers do not treat all the animals in the same way. Farmers tend to treat severe cases differently than first or repeat cases (Samson *et al.*, 2016), and they tend to extend the treatment in moderate compared to mild CM cases (Pinzón-Sánchez and Ruegg, 2011b). Some farmers limit antimicrobial treatment to severe CM (Griffioen *et al.*, 2016), and most farmers are more likely to use antimicrobial to treat high yielding animals or younger animals (Gussmann *et al.*, 2018). This demonstrates that treatment decisions are cow- (Griffioen *et al.*, 2016; Samson *et al.*, 2016) and farm-specific (Oliveira and Ruegg, 2014; Gussmann *et al.*, 2018). Clinical signs, milk conductivity or a failure of an alternative treatment have been reported as the most common sources of information to underpin farmer's antimicrobial treatment decision (Griffioen *et al.*, 2016). Some farmers follow antimicrobial label instructions or a protocol agreed with the veterinarian (Griffioen *et al.*, 2016); others routinely extend treatment beyond the label instructions, built on a social norm belief of "being a good farmer" (Swinkels *et al.*, 2015). These heterogeneities of behaviours are probably associated with feelings of "insecurity" or "uncertainty" that farmers experience regarding CM treatment (Swinkels *et al.*, 2015). These feelings may motivate farmers to seek social approval that "extended treatment is better", which makes it emotionally rewarding (Swinkels *et al.*, 2015). Diagnostic tests can reduce this insecurity and allow for the implementation of evidence-based treatment approaches. Despite this only one third of farmers in the Netherlands reported sending samples to the laboratory and just 2% of the farmers base their treatment decisions on these results (Griffioen *et al.*, 2016). As presented and investigated in previous sections, multiple diagnostics can be used on the farm. However, some of their characteristics do not meet farmers' expectations and preferences. Farmers are interested in advice about treatment and in a result to be delivered in less than 12 hours (Griffioen *et al.*, 2016) as they do not like to postpone treatment decisions (Neeser *et al.*, 2006).

Test characteristics may hinder uptake; however, it seems that farmers are also attached to routine practices and habits, seen as socially accepted norms. Changing these

behaviours and practices is not entirely dependent on the development of new tools, such as those described in Chapters 2 and 3, or on the development of more knowledge, but probably related to other socio-psychological aspects and concepts, where policy development can potentially influence farmers' behaviour to reduce AMU.

5.1.2 Changing practices – behavioural change

To be effective, the available knowledge needs to be communicated and applied. Regarding udder health, communication has been reported as an important tool (Lam *et al.*, 2011; Jansen and Lam, 2012), and if managed ingeniously and tailored to different types of farmers it can allow veterinary practitioners to better support farmers to improve udder health (Lam *et al.*, 2011). In udder health programs, two strategies of communication have been previously identified (Jansen *et al.*, 2010). The central route is supported by different science-based educational tools, but its success depends on farmer's internal motivation (see following section) (Petty and Wegener, 1999). By contrast, peripheral communication is not a science-based strategy, but it can be very effective as a step-by-step approach to change farmer's behaviour (Lam *et al.*, 2011). Both communication types work better in combination and both should be considered to train farmers to implement targeted selective treatment for CM and thereby reduce AMU.

Firstly, farmers need to consider this approach and philosophy relevant (Noar, 2006). A recent study in 71 dairy farms in Wales and England reported that 70% of the farmers consider that reducing AMU would be a "good thing to do" (Jones *et al.*, 2015). To make sure that intentions lead to actions, socio-psychological concepts such as farmers' motivation and mindset are important in the behavioural change process. Motivation can lead to voluntary or involuntary change. Voluntary change can be driven by external motivators, such as economic means such as the application of penalties or premiums with milk price based on milk quality and composition (Múnera-Bedoya *et al.*, 2017). Curiously, penalties for decreased milk quality seemed to work better than premiums in motivating farmers to behavioural change (Valeeva *et al.*, 2007; Huijps *et al.*, 2010). Therefore, the creation of financial penalties regarding AMU above a certain threshold or market restrictions could be a potential way to decrease AMU. Internal motivators can arise from different sources of information, and this type of motivation generally leads to long-term behavioural change. This could potentially be done through global campaigns to increase the awareness of the

impacts of AMU in human and animal health. Compulsory behavioural change is known to be very effective, though it has the limitation that it only works while restrictions are in place (Lam *et al.*, 2011). Implementation of new policies could also work to reduce AMU, for example limiting the number of treatments per animal or per farm. Work from The Netherlands has shown that good udder health can be maintained when restricted antimicrobial use policies are in place (Santman-Berends *et al.*, 2016).

Advisors and veterinarians are in a privileged position to motivate farmers internally through communication. Farmer-led approaches rather than traditional passive knowledge transfer methods have recently been proposed as a good strategy and probably the best way to motivate change (Bard *et al.*, 2017). Motivational interviewing is a communication strategy designed to facilitate clients' internal motivation to change and has been described as an effective way to improve uptake of veterinarian's advice (Bard *et al.*, 2019). Demographic factors and affective attributes, such as a veterinarian's age, respectfulness and dominance, or farmers' education level also influence farmers' satisfaction and willingness to adopt veterinary advice (Ritter *et al.*, 2019). Such insights could be implemented to promote uptake of on-farm diagnostics, targeted treatment and reduce AMU. The regular contacts between veterinarians and farmers build up a relationship of trust and recognise veterinarians as a valuable source of knowledge and advice, therefore veterinarians can be useful to help to detect and understand farmers' mindset which may also influence farmers' behaviour regarding AMU and on-farm diagnostics uptake.

5.1.3 Current mindset

Farmers' mindset involves several psychological concepts such as personality, attitudes, beliefs, values, intentions, skills, knowledge, perceived norms, and perceived self-efficacy. It has been demonstrated that these factors influence farmers' decisions, and are believed to explain to some extent the lack of management changes uptake that would result in noticeable benefits for the farm (Jansen and Lam, 2012). As a result, mindset can affect farm performance (Meek *et al.* 1984; Tarabla and Dodd, 1990; O'Leary *et al.*, 2018), including udder health (Jansen *et al.*, 2009). In the Netherlands, a favourable mindset with regards to the reduction of AMU impacted positively on shifting from a blanket DCT approach to selective DCT (Scherpenzeel *et al.*, 2016), demonstrating the importance of this trait. Thus, understanding farmers' mindset and the specific factors that influence it are

important to motivate farmers to change and to potentially implement targeted selective treatment of CM. Two characteristics of farmers' mindset have been described as fundamental. The first is farmers' need to acknowledge the existence of a problem and their responsibility to take an action. The second is farmers' perception that the recommended strategy is feasible and will be successful on the farm (Ritter *et al.*, 2017). These two characteristics have been described as the main determinants in mastitis management (Jansen and Lam, 2012).

Farmers' perception that the amount of AMU on-farm is high can be a starting point to implement new approaches to control CM with less AMU but does not mean that farmers see it as a problem. Farmers' "normative frame of reference", i.e. the threshold of when an issue becomes a problem, differs between farmers (Jansen and Lam, 2012). It is influenced by their descriptive norms, e.g. how the other farmers treat CM or by injunctive norms, e.g. what practice is approved by other farmers. The use of benchmarking tools to compare AMU between farms (RUMA, 2020) and farm annual AMU reviews with the prescribing veterinarian, as used in food standard schemes in the UK (Red Tractor Assurance, 2018), are examples of strategies to raise farmers' perception of responsibility regarding AMU. These perceptions obviously vary among farmers and are associated with farmers' goals and reasons to reduce AMU. Farmers generally want to reduce AMU to reduce medicine costs (Jones *et al.*, 2015). However, setting a nationwide target to reduce AMU allows the farmer to assess their own reduction against the national benchmark (RUMA, 2020). This is a way to encourage the farmers' sense of being part of a nationwide reduction campaign, as farmers are more likely to assume their responsibility as a joint effort (Brennan and Christley, 2013). Only when farmers identify the amount of AMU as a problem (Jansen *et al.*, 2009), or if they feel responsible for AMR, will they action (Ritter *et al.*, 2017).

The second characteristic is farmers' beliefs in the effectiveness of new mastitis management practices, which is influenced by perceived benefits and barriers to execute them (Jansen and Lam, 2012). Perceived benefits in these situations can be, for example, cows' welfare, economic improvement, compliance with sector-specific AMU targets, long-term job satisfaction, good reputation, consumers' demands, social recognition, pride or desire to conform with perceived standards of "being a good farmer". Perceived barriers can be, for example, extra labour, lack of time, extra economic investments with no financial short-term benefits, uncertainty, etc. (Jansen and Lam, 2012; Ritter *et al.*, 2017). This means

that raising farmers' awareness related to CM blanket treatment costs and consequences and building up farmers' self-confidence and beliefs in the success of a diagnostics-based targeted approach may lead to its implementation and the associated reduction of AMU.

5.1.4 Changing mindset

To change farmers' mindset and behaviour towards the application of a targeted selective treatment, new technical knowledge, new standards, or new perceived norms need to be strong enough to create social pressure, capable to motivate farmers to adopt the desired behaviour. Communication, as stated previously, is a powerful way to inform and diffuse new knowledge and to generate new norms or perceived standards. There are multiple information sources with differences in accessibility, credibility, relevance, practicality, or consistency. Mass media, for example, can reach a broad group of farmers. Tailored approaches, such as participatory learning and one-on-one communication, are very effective in leading to on-farm change (Ritter *et al.*, 2017). One-on-one communication, particularly when done by veterinarians, can be a very powerful way of communicating (Jansen and Lam, 2012). Another important and powerful tool of information is research because evidence-based knowledge can be a way to update beliefs (Higgins *et al.*, 2017), which are another aspect of farmers' mindset. If the new evidence is strong enough, it can result in a belief update to be used for behavioural change, but if the new data differs from present beliefs it may lead to uncertainty and may result in farmers drawing insufficient strength from the evidence or even create scepticism (Higgins *et al.*, 2017). There is evidence that an integrated approach involving stakeholders, udder health specialists, experts in communication, social sciences and marketing can facilitate change of current AMU practices and increase the uptake of diagnostic tests. In addition, creating a new institutional context e.g. milk buyers or food assurance authorities, supported by programs and policies, subsidies, regulations or penalties (Jansen and Lam, 2012), can support and allow a mindset and behavioural change with more prudent AMU. Whilst there is a plethora of new point-of-care diagnostics available (Malcata *et al.*, 2020), their limited uptake to date suggests that more work needs to be done on farmer motivation and behaviour if we want to promote targeted CM treatment and prudent AMU. The specific tests considered in **Chapter 3** and **Chapter 4** were selected to meet farmers' needs in terms of ease of use for informing

treatment decisions (**Chapter 3**) and turn-around time (**Chapter 4**), which are recognized barriers to test uptake (Griffioen *et al.*, 2016).

Conclusion

In this project, I investigated the laboratory-based performance of different tools to support targeted treatment of bovine CM based on differentiation of gram-positive bacteria from other causes of mastitis, using two different approaches.

Using a culture-dependent approach, I evaluated the performance of a simplified test, and I demonstrated that it performs similar to a commercially available on-farm test that was used for benchmarking in terms of sensitivity or accuracy, whilst performing better in terms of specificity. Social science research has shown that farmers are more interested in treatment recommendations than in the knowledge of mastitis pathogens when using on-farm diagnostics, and the simplicity of the slide test can make it an attractive tool for farmers to target antimicrobial treatment of non-severe CM cases caused by gram-positive organisms with good diagnostic accuracy. However, users' willingness, and ability to collect high-quality milk samples were identified as issues that need to be understood and supported. In addition, further work to evaluate the test's user-friendliness and accuracy in on-farm settings, as opposed to the laboratory setting, I used for this initial evaluation, followed by an assessment of uptake, economic impact, and AMU reduction are needed.

To overcome the major limitation of culture-based testing, i.e. it's relatively long turn-around time, I also evaluated a culture-independent approach using a biomarker. The biomarker investigated, cathelicidin, failed to support targeted treatment decisions based on differentiation of gram-positive and other causes of CM. The biomarker showed moderate sensitivity, i.e. it gave a positive result when gram-positive organisms were present, but poor specificity, meaning that it also gave positive results when gram-negative organisms or no growth were present. This combination was responsible for its poor accuracy. In this study, cathelicidin presence was detected using a polyclonal antibody that showed high specificity for mastitis, as cathelicidin was not detected in any of the healthy quarter milk samples. To inform targeted treatment decisions, however, some degree of specificity to differentiate different types of CM is required. Additional work using antibodies that are specific to a subtype of cathelicidin and have been associated with CM caused by gram-positive bacteria

would be a logical next step, as detection of cathelicidin subtypes may be able to inform CM treatment decisions.

In the general discussion, I provided insight into farmers' behaviour and mindset regarding current CM treatment practices and the barriers and drivers for uptake of on-farm diagnostics. Current CM treatment practices vary between countries, and are herd- and cow-specific, and despite the evidence available to support targeted treatment of CM and available on-farm diagnostic tests, its uptake is limited or unquantified in most countries. Several barriers such as test characteristics, farmers' routine practices, labour shortages, lack of time, cost and uncertainty regarding the tests' financial benefit, were identified as possible barriers that limit diagnostics uptake. On the other hand, potential economic benefits, social recognition, pride, improved cow welfare, and the perception of being a good farmer were identified as potential drivers for their uptake. Mindset, which includes farmer's attitudes, beliefs, perceived norms and perceived self-efficacy, influences behavioural change and has been recognised as an important feature in the implementation of mastitis control programs. An integrated approach with multiple stakeholders, supported with potential policies, subsidies, strategies of AMU benchmarking and communication, may help to raise knowledge and awareness about AMU and may help to change farmer's mindset and encourage on-farm diagnostics and targeted selective treatment uptake.

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Appendices

Appendix 1: Laboratory-based evaluation of a simplified point-of-care test intended to support treatment decisions in non-severe bovine clinical mastitis

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Abstract

To limit the use of antimicrobials in dairy cattle, farmers are increasingly encouraged to adopt targeted treatment decisions based on knowledge of the pathogens causing clinical mastitis (CM), whereby treatment of non-severe CM is generally recommended for gram-positive mastitis but not for gram-negative or culture-negative mastitis. The objectives of this study were to conduct a laboratory-based evaluation of the performance of a simplified slide test as a tool to differentiate gram-positive CM from other cases of CM, and to compare its performance against a commercially available on-farm test that is commonly used in our area (VétoRapid). Test outcomes after 24-48 hr incubation were compared to results from bacteriological culture and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Milk samples (n = 156) were obtained from cases of severe and non-severe CM on seven farms and collected by farm personnel. After removal of contaminated samples (n = 23) and organisms with unknown species identity (n = 3), the simplified slide test showed high sensitivity and accuracy (> 80%), similar to the comparator test. For most outcomes of interest (culture positive, Escherichia coli, or gram-positive growth), the specificity of the slide test (85.7, 97.5 and 76.8% respectively) was higher than the specificity of the comparator test (57.1, 92.4 and 58% respectively). When considering non-severe cases of CM only, and interpreting detection of gram-positive organisms as indicative of the need for antimicrobial treatment, the simplified test had higher specificity (77.4% vs 60.4%) and higher positive predictive value (79.7% vs 70.0%) than the comparator test and similar sensitivity (83.9% vs 87.5%). The proportion of sampled CM cases, contaminated samples and gram-positive mastitis cases - which affects the positive and negative predictive value, the economic value of diagnostic testing and its potential to reduce antimicrobial use - differed between farms. The simplicity and accuracy of the slide test could make it an attractive tool for farmers to target antimicrobial treatment of non-severe clinical mastitis.

Keywords: point-of-care test, bovine clinical mastitis, treatment, antimicrobial use

Mastitis control is the most frequent reason for antibiotic use in lactating and non-lactating dairy cows (Pol & Ruegg, 2007). Because of concerns that antibiotic usage may lead to antimicrobial resistance (WHO, 2015), strategies are needed to promote and ensure prudent use of antimicrobials for mastitis control. Selective treatment for CM in lactating cows, based on differentiation between gram-positive pathogens and other causes of mastitis, has the potential to reduce antibiotic use significantly without negative impact on udder health, production or culling (Lago *et al.* 2011a, b; Mansion-de Vries *et al.* 2014). This has led to development of an array of diagnostic tools for on-farm classification of mastitis pathogens to support selective treatment.

Numerous culture-based detection kits for classification of mastitis pathogens have been reviewed, and new tests are becoming commercially available (Malcata *et al.* 2020). There are diagnostic tests based on Petrifilm, agar plates, or tube-test based systems. Some identify bacteria as gram-positive or gram-negative, whereas other tests identify bacteria to genus or species-level (Malcata *et al. 2*020). Some assays also include antibiotic susceptibility testing (Jones *et al.* 2019). All tests are more reliable when used for diagnosis of broad categories, such as growth, gram-positive and gram-negative species, rather than at genus or species level (Lago & Godden, 2018). The performance of diagnostic assays can be evaluated using scientific characteristics such as sensitivity, specificity and accuracy, and convenience aspects such as cost, ease of use and turn-around time. For example, most Petrifilm or agar-based tests are cheap but require considerable user training whereas

Mastatest (Jones *et al.* 2019) costs more but provides automated reading to increase ease of use.

Although many tests were designed to identify pathogens to genus or species level, farmers are more interested in advice on antibiotic use than identification of causative agents of CM (Griffioen *et al*. 2016). A simplified test to differentiate gram-positive organisms from other causes of mastitis could be sufficient to decide whether antimicrobial treatment of non-severe CM is needed. In a different context, namely bacteriuria in pregnant women, a similar need for reliable and simple testing to differentiate gram-positive, gram-negative and culture-negatives samples led to development of the Uricult dip-slide (Van Dorsten & Bannister, 1986). The dip-slide is a plastic paddle with two selective media that can be dipped in a liquid sample such as urine or milk, allowing for growth of either gram-positive or gram-negative organisms.

The aim of our study was to evaluate the laboratory performance of a simplified slide test for bovine mastitis, to determine whether it differentiates gram-positive from other forms of mastitis with similar accuracy as a commercially available comparator test commonly used in our practice.

Material & Methods

Regulatory compliance

This research was approved by the Ethics and Welfare Committee, School of Veterinary Medicine, University of Glasgow, UK (Ref 50a/16).

Sample collection

Seven dairy farms in Scotland were selected based on herd size, location, and willingness to cooperate in the study (Supplemental Table S1). Farm staff, including milkers and herd managers, were trained to identify CM and to classify cases as mild (abnormal milk, e.g. clots, flakes or serous milk), moderate (abnormal milk and signs of udder inflammation: hardness, swelling, redness, heat or pain) or severe (presence of additional systemic signs of disease, e.g. fever, tachycardia, tachypnea, dehydration, or anorexia) (Pinzón-Sánchez & Ruegg, 2011). They were taught how to collect milk samples aseptically according to National Mastitis Council recommendations (NMC, 2017). CM cases were sampled regardless of mastitis severity. If multiple quarters of a cow were affected simultaneously, each affected quarter was sampled. Any CM episode in a quarter occurring >14 days after the previous episode, or caused by a different etiological agent, was considered a new CM case. Animals were eligible for inclusion in the first week after calving but no animals included were within 14 days of administration of antimicrobial products.

Samples were collected from January to May 2018. They were stored on farm at -20°C, and transported once a week to Glasgow University's Veterinary Diagnostic Services laboratory where they were stored at -20°C until processing. All samples were cultured within 4 weeks from CM detection.

Reference test

Samples were thawed at ambient temperature for up to 8 hours and processed simultaneously using the reference test, the simplified slide test, and the commercially available plate-based comparator test as described in the following two sections. For consistency, all media were inoculated and read by the first author, starting with the slide

test. Bacteriological culture (NMC, 2017) with subsequent determination of species identity using MALDI-ToF MS was used as the reference test as detailed in the supplementary file.

Slide test

Media of the simplified slide test (VétoSlide, Vétoquinol, Lure, France) were inoculated by applying milk directly to each side using cotton wool swabs (approximate volume 0.1 ml) to moisten the entire surface of the media, as per manufacturer's instructions. The inoculated slides were incubated aerobically at 37° and examined after 24-48 hours. When at least one colony was visible, the sample was considered positive (Dohoo *et al.* 2011). Based on the manufacturer's guidelines, any growth on the green media was considered to indicate presence of gram-negative bacteria and red colonies on the green media were considered *Escherichia coli*. Growth on the red media was considered to indicate presence of gram-positive bacteria (Supplemental Figure S1). When there was growth on both media, it was considered to indicate mixed infection with gram-positive and gram-negative bacteria. Guidelines to identify contaminated samples were not given, so samples were never classed as contaminated based on the slide test.

Comparator test

Plates for the comparator test (VétoRapid, Vétoquinol, Lure, France) were chosen for benchmarking because it is the most commonly used on-farm test in the dairy community of the authors and was previously evaluated in similar study settings (Viora *et al.* 2014). These plates were inoculated with 0.01 ml of milk per sector using disposable sterile calibrated plastic loops, incubated aerobically at 37°C and examined after 24-48 hours, as detailed in the supplementary file. Results were summarized as gram-positive, gram-negative, *E. coli* and no growth for comparison with the reference and slide tests results. Samples not yielding visible colonies on the comparator test were considered negative for mastitis-associated pathogens. As for the slide test, a contaminated category was not specified by the manufacturer.

Data analysis

Samples that were contaminated or contained non-identifiable isolates by MALDI-ToF MS were excluded from evaluation of diagnostic test performance. All other culture-positive and culture-negative samples (n = 130) were used to calculate sensitivity, specificity, accuracy, positive predictive values (PPV) and negative predictive values (NPV) for growth, gram-positives, gram-negatives and *E. coli*. The reference test was used to classify results from the slide test and comparator test as correct or incorrect. To evaluate the potential of the test kits as treatment decision support tools, the calculations were repeated using a subset of the 130 samples, namely those from non-severe CM cases (n = 109), and the outcome was expressed as "treatment". This outcome is equivalent to gram-positive growth or no gram-positive growth. The latter category includes gram-negative bacteria, non-bacterial growth, and culture-negative results.

Statistical analysis was performed in Excel (Microsoft Corp., Redmond, USA) using tabular methods, and in R. If the 95% confidence interval for the difference between tests excluded zero, test performance was considered significantly different. Full details are provided in the supplementary file.

Results

Reference test

Per farm, 5 to 58 samples were collected (Supplemental Table S1). Of 156 samples, 23 (14.7%) were contaminated. Among 133 non-contaminated samples, 14 (10.5%) showed no growth, and 116 (87.2%) showed growth of one or two colony types that could be identified by the reference method (Table 1). Three samples with growth of organisms that could not be identified by the reference method, were excluded from further analysis. Within each farm's sample set, gram-positive and gram-negative isolates were identified, with a preponderance of gram-positive results for some farms (Farms 3, 4 and 5), mostly gram-negative results for others (Farms 2 and 6) and an even balance for the remainder (Farms 1 and 7; Figure 1). The proportion of contaminated samples per farm ranged from 0 to 33%, indicating considerable differences in sample quality. The most common species were *E. coli* and *S. uberis*, followed by other major mastitis pathogens, including *S. dysgalactiae*, *S. aureus* and *Klebsiella* (Table 1). *(Figure 1 near here)*

Slide test

After excluding contaminated samples and those with unidentified organisms, 130 samples were used to evaluate the performance of the slide test. A milk sample could be culture negative or culture positive, contain a single colony type or two colony types (two gram- positive morphotypes, two gram-negative morphotypes, or mixed gram-positive and gram-negative growth). The latter were considered gram-positive in the gram-positive analysis and gram-negative in the gram-negative analysis. The proportion of culture negative results was considerably higher for the slide test (20%) than for the reference test (10.8%). Of 26 culture-negative samples in the slide test, 12 (46.2%) were correctly classified. Of 14 false negative slide test results, seven were from samples with gram-positive growth in the reference test and seven from samples with gram-negative growth.

Mixed gram-positive and gram-negative growth was more common in the slide test (12.3%) than in the reference test (5.4%) (Table 1). *(Table 1 near here)*

The sensitivity of the slide test was similar for gram-positive and gram-negative organisms, but specificity was higher for the latter, resulting in higher accuracy for gram-negative organisms (89.2%) or *E. coli* (92.3%) than for gram-positive organisms (79.2%), (Table 2). For the samples from non-severe CM (n = 109), the potential of the slide test to be used as treatment decision support tool was evaluated. Sensitivity and specificity for this subset were similar to those for all CM cases (Table 2).

Mean PPV of the slide test was high (between 92.6 and 98.1%) for growth, gram-negative results, and *E. coli* and moderate (between 75 and 80%) for gram-positive results (all based on 130 samples) and treatment (based on 109 samples). The mean NPV was less than 50% for growth and between 82 and 90.6% for all other outcomes (Table 2). *(Table 2 near here)*

Benchmarking against the comparator test

Despite using a higher inoculum, the slide test gave fewer false positive results for growth than the comparator test, resulting in significantly higher specificity. Low specificity of the comparator test was partly due to moderate specificity in the detection of *S. aureus* and *S. uberis* (70.7% and 82.6%, respectively; Supplemental Table S2), which was accompanied by high sensitivity for the same pathogens (100% and 90.5%, respectively). For sensitivity, there were no statistically significant differences among tests for any of the outcomes (Table 2). Despite the greater specificity of the slide test, overall accuracy of the two tests was not significantly different. In our study population, the slide test had significantly greater PPV than the comparator test for growth, gram-positive, *E. coli* and treatment, whereas their NPVs were similar for all outcomes.

Discussion

We evaluated the laboratory performance of a simplified culture-based slide test, VétoSlide, which was developed as a potential point-of-care tool to support farmers' CM treatment decisions. Its accuracy for gram-positive organisms in samples from non-severe CM (80.7%) is in the same range as commercially available point-of-care tests, including the comparator test (VétoRapid) (74.3%, this study), the Minnesota Easy Culture System-Triplate (81.3%) (Ferreira *et al.* 2018), Minnesota Easy Culture System-biplate (81 to 84%) (Royster *et al.* 2014), Petrifilm (80.2%) (Mansion-de Vries *et al.* 2014) and MastDecide (58.6 to 85.3%) (Leimbach & Krömker, 2018), although comparisons between studies are complicated by differences in study design, populations, and methods of analysis. Such differences make comparison of predictive values problematic because they are highly dependent on pathogen prevalence, which are farm-specific. Benchmarking of the new slide test against a commercially available comparator in a single study allowed us to compare predictive values, which are more important in practice than sensitivity, specificity, or accuracy. A high PPV means that unnecessary treatment is minimised whereas a high NPV means that treatment is withheld only when cows truly do not need it. The slide test outperformed the comparator test in PPV and had similar NPV, meaning that the reduced risk of over-treating was not accompanied by an increased risk of under-treating. Whether positive or negative predictive value is considered more important in informing treatment decisions differs between regions. In some areas, such as southern Europe, it is generally assumed that antimicrobial treatment of mastitis is needed until proven otherwise (Busani *et al.* 2004). Conversely, in northern Europe, it is assumed that treatment is not needed until proven otherwise (Persson Waller *et al.* 2016). Within countries, this balance may shift over time, as illustrated by work from The Netherlands on selective dry cow treatment (DCT). Two split-udder trials conducted two decades apart (Schukken *et al.* 1993; Scherpenzeel *et al.* 2014) in the same country both showed that blanket DCT prevents CM when compared to selective DCT. However, the first study concluded that blanket DCT should be used to prevent CM despite the need to "eliminate unnecessary use of antibiotics"; whereas the second study emphasized the reduction in antimicrobial use that could be achieved by abandoning blanket DCT.

Whether the price of the diagnostic test is worth paying in terms of financial benefit is a matter of debate. On farms with a single dominant pathogen, the value of information may be limited (Cha *et al.* 2016), but our data showed that several farms did not have clear predominance of gram-positive or gram-negative mastitis over other types of mastitis. Some authors argue that even with just 20% of gram-positive mastitis, use of on-farm diagnostics would not be cost-effective (Down *et al.* 2017). Hence, the value of information would be farm-specific and no blanket statements around cost-benefit or reductions in antimicrobial use can be made based on our results. It is clear, however, that some farms will need further training in sample collection and handling to reduce the number of contaminated samples and to make investment of time and money into diagnostic testing better value for money. Moreover, before uptake of the slide test can be recommended on-farm, evaluation under on-farm conditions will be needed, as our laboratory-based analysis included freezing and thawing of milk, which would not be part of its on-farm use. Reading of plates at 24 and 48 hrs, as done here to allow for comparison with VétoRapid results, would cause considerable delay in treatment decisions, and shorter incubation times would need to be considered, with growth of gram-negative organisms often visible well within 24 hrs (data not shown).

The reference test used in our study included species identification by MALDI-ToF MS and revealed the presence of several species that are not recognized as typical mastitis

pathogens, e.g. *Bacillus* and *Lysinibacillus* species. Although both genera are gram-positive, it is debatable whether they should be targeted with antimicrobial treatment because little is known about their role as pathogenic agents or their response to treatment. None of the currently available point-of-care tests for mastitis have the ability to differentiate such organisms from recognized mastitis pathogens. When information at species or subspecies level is required for advanced investigations or decision making at herd or animal level, laboratory-based microbial diagnostics continue to be important (Mansion-de Vries *et al.* 2014). For on-farm treatment decision making, however, the simplified slide test appears to have the potential to be an affordable, accurate, and user-friendly option.

Conclusion

Using laboratory-based evaluation of farmer collected milk samples, we demonstrated that a simplified slide test performs similar to the commercially available on-farm test that was used for benchmarking in terms of sensitivity or accuracy, whilst performing better in terms of specificity. The simplicity of the slide test can make it an attractive tool for farmers to target antimicrobial treatment of non-severe CM cases caused by gram-positive organisms with good diagnostic accuracy. Further evaluation of user-friendliness, and test accuracy in on-farm settings is needed, followed by assessment of uptake, economic impact, and reduction in antimicrobial use. In addition, users' willingness and ability to collect high quality milk samples needs to be understood and supported.

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Table legends:

Table 1:

Test results of 130 milk samples from bovine clinical mastitis based on a reference test consisting of standard bacteriological culture and species identification by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS) in comparison with the simplified slide test under evaluation (VétoSlide, Vétoquinol, Lure, France) and a commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France).

Table 2:

Performance of the simplified slide test under evaluation (VétoSlide, Vétoquinol, Lure, France) and a commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) for identification of mastitis pathogens (n = 130 samples) and as a treatment decision support tools for non-severe clinical mastitis (n = 109 samples). Results are based on comparison with a reference test consisting of culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry and the difference compares the two tests while correcting for dependence. Values are point estimates expressed as percentages, with 95% confidence intervals in brackets.

Supplemental Table S1:

Farms participating in sample collection to evaluate potential on-farm diagnostics for clinical mastitis in dairy cattle.

Supplemental Table S2:

Performance of the commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) for identification of mastitis pathogens (n = 130 samples) based on comparison of results at species, genus or group level with a reference test consisting of non-selective culture and species identification based on matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry. Values are point estimates expressed as percentages, with 95% confidence intervals in brackets.

Figure legends:

Figure 1:

Sampling results for bovine milk samples (n = 130) from quarters with clinical mastitis by participating farm. The number of samples for each farm were, from farm 1 to 7 respectively, 58, 30, 35, 5, 8, 12 and 10.

Supplemental Figure S1:

Simplified slide test (VétoSlide, Vétoquinol, Lure, France) and commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) for on-farm mastitis diagnostics. (A) The green media on the slide allow for detection of gram-negative bacteria. (B) *Escherichia coli* forms red colonies (right panel) and other gram-negative bacterial species form white colonies (left panel). (C) The red media allows for detection of gram-positive bacteria. (D) Bacterial growth on red media. E. Commercially available plate-based comparator (VétoRapid, Vétoquinol, Lure, France) with 3 selective indicator media. (F) Growth in the sector selective for gram-negative bacteria. (G) Growth in the sector selective for staphylococci. (H) Growth in the sector selective for streptococci and enterococci.

Table 1:

Result	Reference Number[1] (%)	VétoSlide Number[1] (%)	VétoRapid Number[1] (%)
No growth	14 (10.8)	26 (20.0)	17 (13.1)
Growth	116 (89.2)	104 (80.0)	113 (86.9)
Gram-positive only	54 (41.5)	50 (38.5)	55 (42.3)
Gram-negative only	53 (40.8)	38 (29.2)	30 (23.1)
Mixed (gram-positive & gram-negative)	7 (5.4)	16 (12.3)	28 (21.5)
Gram-negative	60 (46.2)	54 (41.5)	58 (44.6)
Escherichia coli	51 (39.2)	45 (34.6)	50 (38.5)
Klebsiella spp.	6 (4.6)	n/a	16 (12.3)
Other gram-negative	4 (2.8)	n/a	2 (1.5)
Gram-positive	61 (46.9)	66 (50.8)	83 (63.8)
Staphylococcus spp.	21 (16.2)	n/a	56 (43.1)
Staphylococcus aureus	7 (5.4)	n/a	43 (33.1)
Non-aureus staphylococci	15 (11.5)	n/a	21 (16.2)
Streptococcus spp.	32 (24.6)	n/a	50 (38.5)
Streptococcus dysgalactiae	10 (7.7)	n/a	11 (8.5)
Streptococcus uberis	21 (16.2)	n/a	38 (29.2)
Other *Streptococcus* spp.	1 (0.8)	n/a	6 (4.6)
Enterococcus spp.	3 (2.3)	n/a	2 (1.5)
Other gram-positive	13 (10.0)	n/a	1 (0.8)
Prototheca zopfii	2 (1.5)	n/a	n/a

[1]The total number of species/genera listed exceeds the number of samples because more than one species/genus was detected in some samples that were not contaminated based on the NMC standard definition of 3 or more colony types (the percentage shown is related to the proportion of samples). n/a = not applicable.

Table 2:

	VétoSlide	VétoRapid	Difference
Sensitivity			
Growth	87.9 [80.8; 92.7]	92.2 [85.9; 95.9]	4.3 [-2.2; 10.8]
Gram-negative	83.3 [72.0; 90.7]	83.3 [72.0; 90.7]	0.0 [-9.2; 9.2]
Gram-positive (n = 61)	82.0 [70.5; 89.6]	88.5 [78.2; 94.3]	6.6 [-3.5; 16.6]
E. coli (n = 51)	84.3 [72.0; 91.8]	86.3 [74.3; 93.2]	2.0 [-9.6; 13.5]
Treatment (n = 56)	83.9 [72.2; 91.3]	87.5 [76.4; 93.8]	3.6 [-6.3; 13.4]
Specificity			
Growth	85.7 [60.1; 96.0]	57.1 [32.6; 78.6]	-28.6 [-52.2; -4.9]*
Gram-negative	94.3 [86.2; 97.8]	88.6 [79.0; 94.1]	-5.7 [-13.5; 2.1]
Gram-positive	76.8 [65.6; 85.2]	58.0 [46.2; 68.9]	-18.2 [-30.4; -7.3]*
E. coli	97.5 [91.2; 99.3]	92.4 [84.4; 96.5]	-5.1 [-9.9; -0.2]*
Treatment	77.4 [64.5; 86.5]	60.4 [46.9; 72.4]	-17 [-30.6; -3.4]*
Accuracy			
Growth	87.7 [80.9; 92.3]	88.5 [81.8; 92.9]	0.8 [-5.8; 7.3]
Gram-negative	89.2 [82.7; 93.5]	86.2 [79.2; 91.1]	-3.1 [-9.1; 2.9]
Gram-positive	79.2 [71.5; 85.3]	72.3 [64.1; 79.3]	-6.9 [-15; 1.1]
E. coli	92.3 [86.4; 95.8]	90.0 [83.6; 94.1]	-2.3 [-7.7; 3.1]
Treatment	80.7 [72.3; 87.0]	74.3 [65.4; 81.6]	-6.4 [-15; 2.1]
Positive predictive value			
Growth	98.1 [93.3; 99.5]	94.7 [88.9; 97.5]	-3.4 [-6.7; -0.03]*
Gram-negative	92.6 [82.4; 97.1]	86.2 [75.1; 92.8]	-6.4 [-15.2; 2.4]
Gram-positive	75.8 [64.2; 84.5]	65.1 [54.3; 74.4]	-10.7 [-18.9; -2.55]*
E. coli	95.6 [85.2; 98.8]	88.0 [76.2; 94.4]	-7.6 [-14.8; -0.3]*
Treatment	79.7 [67.7; 88.0]	70.0 [58.5; 79.5]	-9.7 [-18.5; -0.86]*
Negative predictive value			
Growth	46.2 [28.8; 64.5]	47.1 [26.2; 69.0]	0.9 [-18.7; 20.5]
Gram-negative	86.8 [77.4; 92.7]	86.1 [76.3; 92.3]	-0.7 [-7.3; 5.8]
Gram-positive	82.8 [71.8; 90.1]	85.1 [72.3; 92.6]	2.3[-7.3; 11.9]
E. coli	90.6 [82.5; 95.2]	91.3 [83.0; 95.7]	0.7 [-5.8; 7.2]
Treatment	82.0 [69.2; 90.2]	82.1 [67.3; 91.0]	0.1 [-10.7; 10.8]

*Outcomes where VétoSlide and VétoRapid are significantly different

The total of samples that yield in the reference test growth, Gram-negative, Gram-positive, E. coli and Treatment were, 116, 60, 61, 51 and 56, respectively.

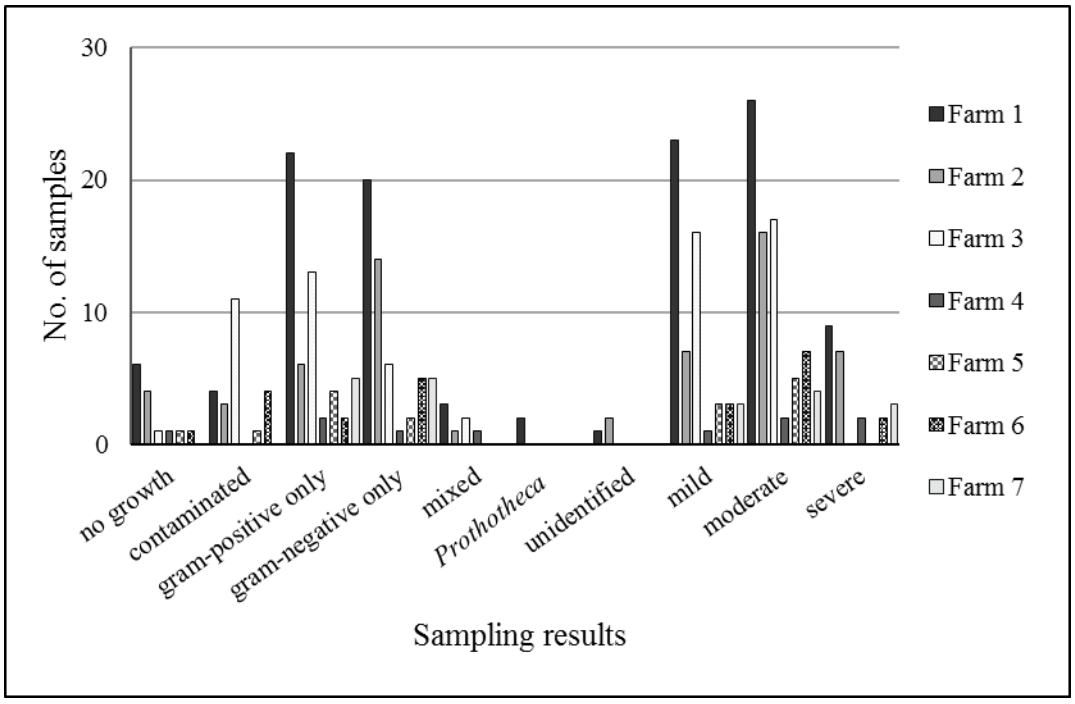
Supplemental Table S1:

Farm	Lactating cows (n)	Samples collected (n)	Bulk tank SCC (×1000 cells/ml)	Clinical mastitis incidence per 100 cows per year	Milking Frequency (/day)	Milking parlor type	Bedding
1	795	58	140	28	3	52-point rotary	sand
2	549	30	180	30	3	24 x 48 SwingOver herringbone	oat husks and lime
3	496	33	230	60	3	48 x 48 herringbone	sawdust and lime
4	607	5	145	40	3	24 x 48 SwingOver herringbone	recycled manure solids
5	304	8	195	45	2	24 x 24 herringbone	oat husks and lime
6	296	12	280	65	2	24 x 48 SwingOver herringbone	sawdust and lime
7	276	10	190	45	2	44-point rotary	sawdust and lime

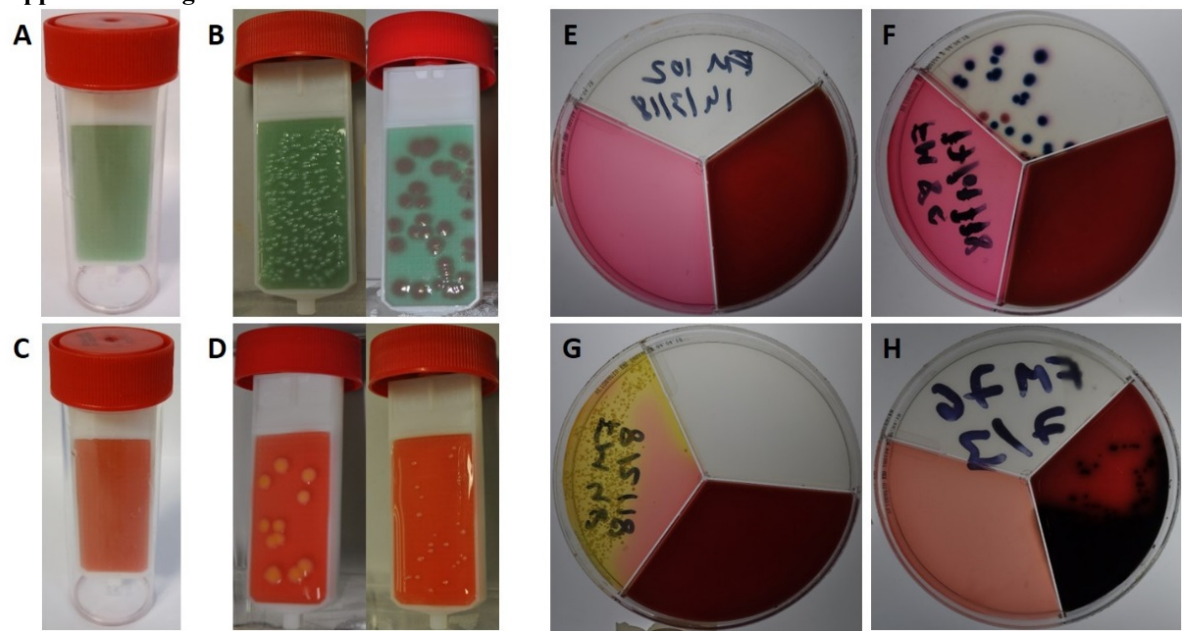
Supplemental Table S2:

Epidemiological parameter	Gram-positive						Gram-negative		
	Staphylococcus aureus (n = 7)	Non-aureus Staphylococci (n = 15)	*Streptococcus dysgalactiae* (n = 10)	*Streptococcus uberis* (n = 21)	*Streptococcus agalactiae* (n = 0)	*Enterococcus* spp. (n = 3)	*Escherichia coli* (n = 51)	*Klebsiella* spp. (n = 6)	Other gram-negative (n = 4)
True prevalence	5.4 [2.6; 10.7]	11.5 [7.1; 18.2]	7.7 [4.2; 13.6]	16.2 [10.8; 23.4]	0 [0; 2.9]	2.3 [0.8; 6.6]	39.2 [31.3; 47.8]	4.6 [2.1; 9.7]	3.1 [1.2; 7.6]
Apparent prevalence	33.1 [25.6; 41.5]	16.2 [10.8; 23.4]	8.5 [4.8; 14.5]	29.2 [22.1; 37.6]	4.6 [2.1; 9.7]	1.5 [0.4; 5.4]	38.5 [30.5; 47]	9.2 [5.4; 15.4]	0.8 [0; 4.2]
Accuracy	72.3 [64.1; 79.3]	81.5 [74.0; 87.3]	93.1 [87.4; 96.3]	83.8 [76.6; 89.2]	95.4 [90.3; 97.9]	96.2 [91.3; 98.3]	90.0 [83.6; 94.1]	92.3 [86.4; 95.8]	96.2 [91.3; 98.3]
Sensitivity	100 [64.6; 100]	40.0 [19.8; 64.3]	60.0 [31.3; 83.2]	90.5 [71.1; 97.3]	not applicable	0 [0; 56.1]	86.3 [74.3; 93.2]	66.7 [30.0; 90.3]	0 [0; 49.0]
Specificity	70.7 [62.2; 78.0]	87.0 [79.6; 91.9]	95.8 [90.6; 98.2]	82.6 [74.4; 88.5]	95.4 [90.3; 97.9]	98.4 [94.4; 99.6]	92.4 [84.4; 96.5]	93.5 [87.8; 96.7]	99.2 [95.6; 100]
Positive predictive value	16.3 [8.1; 30.0]	28.6 [13.8; 50.0]	54.5 [28.0; 78.7]	50.0 [34.8; 65.2]	0 [0; 39.0]	0 [0; 65.8]	88.0 [76.2; 94.4]	33.3 [13.8; 60.9]	0 [0; 94.9]
Negative predictive value	100 [95.8; 100]	91.7 [85.0; 95.6]	96.6 [91.7; 98.7]	97.8 [92.4; 99.4]	100 [97.0; 100]	97.7 [93.3; 99.2]	91.3 [83.0; 95.7]	98.3 [94.0; 99.5]	96.9 [92.3; 98.8]

Figure 1:



Supplemental Figure S1:



Appendix 2: Point-of-care tests for bovine clinical mastitis: What do we have and what do we need?

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Abstract

Mastitis, inflammation of the bovine mammary gland, is generally caused by intramammary infection with bacteria, and antimicrobials have long been a corner stone of mastitis control. As societal concern about antimicrobial use in animal agriculture grows, there is pressure to reduce antimicrobial use in dairy farming. Point-of-care tests for on-farm use are increasingly available as tools to support this. In this Research Reflection, we consider available culture-dependent and culture-independent tests in the context of ASSURED criteria for low-resource settings, including convenience criteria, scientific criteria and societal criteria that can be used to evaluate test performance. As tests become more sophisticated and sensitive, we may be generating more data than we need. Special attention is given to the relationship between test outcomes and treatment decisions, including issues of diagnostic refinement, antimicrobial susceptibility testing, and detection of viable organisms. In addition, we explore the role of technology, big data and people in improved performance and uptake of point-of-care tests, recognising that societal barriers may limit uptake of available or future tests. Finally, we propose that the 3Rs of reduction, refinement and replacement, which have been used in an animal welfare context for many years, could be applied to antimicrobial use for mastitis control on dairy farms.

Keywords: Dairy, clinical mastitis, point-of-care, antimicrobial, diagnostics

Setting the Scene: Background and Aim

Mastitis is an inflammation of the mammary gland, frequently caused by intramammary infections (IMI), and occasionally by mechanical or chemical trauma (through teat or udder trampling for instance), or when cleaning liquids are mistaken for teat disinfectants. Mastitis and IMI are different biological processes that require different diagnostic tools and treatments. Mastitis is characterized by physical and chemical changes in milk and, in moderate or severe cases, by pathological changes in the mammary gland or systemically. Clinical mastitis (CM) can be detected using human senses (vision, touch, and taste, although the latter is discouraged), whereas detection of subclinical mastitis (SCM) requires additional tools. With no one-to-one relationship between clinical severity and causative agent, the cause of mastitis can rarely be detected without further testing. Ideally, mastitis treatment is based on knowledge of severity, causative agent and prognosis so that anti-inflammatory, antimicrobial and supportive treatments can be selected for maximum efficacy and minimal side effects, which include potential selection for antimicrobial resistance (AMR). The contribution of antimicrobial use (AMU) to selection for AMR has become of concern due to actual or predicted adverse effects in human and veterinary health (O'Neill, 2016). Both quantity and "quality" (or type) of antimicrobials are important. The World Health Organization (WHO) has identified Highest Priority Critically Important Antimicrobials (HP-CIA) for human medicine (WHO, 2019), and suggested that HP-CIA "should not be used for treatment of food-producing animals with a clinically diagnosed infectious disease" (WHO, 2017). Quinolones, macrolides and ketolides, polymixins and 3rd and 4th generation cephalosporins are all HP-CIA used for mastitis treatment and we can do better in this area (Turner *et al.* 2018; Doehring & Sundrum, 2019).

Selective treatment is the practice of restricting AMU to cases most likely to benefit from treatment and may help to reduce AMU. The phrase "targeted treatment" is also used and hints at a different underlying philosophy. "Selective treatment" suggests blanket treatment as

the default option, which is modified by selecting cows for treatment. Targeted treatment suggests no treatment to be the default option, with targeting of treatment to those cows that are most likely to benefit. Whilst selective dry cow treatment has been or become the norm in several countries (Vanhoudt *et al*. 2018), selective treatment of lactational mastitis is a more recent development (Lago & Godden, 2018). Principles underpinning this approach include the notion that AMU must be reduced in livestock; the suggestion that HP-CIA should be avoided in veterinary medicine; the recognition that mild to moderate CM caused by gram-negative pathogens does not necessarily need antimicrobial treatment; evidence that narrow-spectrum antimicrobials are effective against most gram-positive mastitis; and the maxim that "lack of an indication is a contra-indication", whereby absence of viable bacteria may be interpreted as lack of an indication. Each of these principles is subject to debate, to individual differences in weighing of available evidence, and to technical or societal opportunities and constraints, which explains the wide range of AMU practices on dairy farms. Central to targeted treatment of lactational CM is the ability to differentiate mild-to-moderate gram-positive mastitis from other forms of mastitis. Point-of-care (POC) testing can make this distinction. For the sake of this paper, we interpret "point-of-care" as a tool to inform treatment decisions. Generally, this requires on-farm testing, although some commercial diagnostic laboratories or veterinary practices may offer a 24-hr turn-around time (TAT) or service.

In this paper, we reflect on characteristics of POC tests with a focus on CM diagnostics that can inform treatment decisions. Specifically, we focus on how such tests succeed or fail in addressing professional and societal needs, and how future developments might change this.

What do we need?

We need to detect CM cases, which is usually done during milking, by people or by equipment (reviewed by Hogeveen *et al.* 2010). Where broad-spectrum antimicrobials are advocated for treatment of CM, one could argue that there is no need for further diagnostics. To reduce AMU, however, treatment would need to be targeted to a subset of CM cases based on severity and causative agent. In addition to reduction in AMU, refinement may be possible, e.g. by avoidance of HP-CIA antimicrobials, by use of narrow-spectrum antimicrobials, or possibly based on antimicrobial susceptibility of pathogens. Many professionals and scientists accept that pathogens such as *Mycoplasma* spp. and non-bacterial organisms such as algae, (*Prototheca* spp.) and yeast (e.g. *Candida* spp.) will not respond to antimicrobial treatment and that mild to moderate gram-negative mastitis does not need to be treated with antimicrobials (Lago & Godden, 2018). Some will also argue that culture-negative mastitis cases are unlikely to respond to antimicrobial treatment because there are no viable bacteria to treat, whereas others emphasise that culture results may be false negative. Conversely, detection of gram-positive bacteria is not necessarily indicative of IMI as it may result from contamination of samples or tests, or from presence of clinically non-significant organisms (Nyman *et al.* 2016). Here, we discuss ways to assess diagnostic test performance in relation to diagnostic needs before presenting detail of available tests in subsequent sections.

Convenience criteria

In terms of diagnostic capability, farms are resource-constrained settings. To evaluate POC test performance in resource-constrained human health care settings, The ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) were developed (Kosack *et al.* 2017). They capture scientific criteria

(sensitivity, specificity) and convenience criteria ("cheap", "easy", "quick"), and will be used here as framework for discussion of POC tests for CM.

Dairy production is a sector which often has low profit margins, which might seem to argue for tests with low costs, but price is not necessarily a priority (Griffioen *et al.* 2016). Cost may need to be balanced against ease of use, whereby some of the cheapest systems may require significant user training and hence staff retainment. More expensive systems may have higher ease of use due to automated sample processing, reading or interpretation and may be more robust to changes in environment or personnel. There may also be trade-offs between cost and time. For example, culture-based diagnostics inherently have a slow turn-around due to the time needed for bacterial growth, but they can be very affordable. Conversely, currently available DNA-based diagnostics are fast but require expensive instrumentation. The relative costs of reagents, equipment and labour may be quite different between countries, a phenomenon that is also reflected in the predominance of milking systems (hand milking, milking parlour, or automated milking system) in different parts of the world. Whether low-tech or high-tech, good diagnostic tests cannot compensate for poor quality samples. Any test applied to a contaminated sample is a waste of resources. Surprisingly, not every POC test has "contamination" as a recognized outcome. This may mask the presence of false positives and give high test sensitivity at the expense of test specificity, potentially leading to "justification" of AMU based on detection of contaminants rather than mastitis pathogens. Sample quality, although rarely discussed, may be more important than test characteristics.

Scientific criteria

Diagnostic tests are routinely judged based on the scientific criteria of sensitivity ("ability to recognise positives") and specificity ("ability to recognise negatives"). Tests should be validated in the host species and under the conditions where they are intended to be applied but most published POC test evaluations are laboratory-based rather than farm-based (Table 1). Estimates of scientific criteria are often obtained by comparing the test results with a reference test or "gold standard". Because no test is perfect, no-gold standard comparison or latent class analysis is also used (Nyman *et al.* 2016). Outcomes of interest can be presence versus absence or quantitative thresholds, such as counts of colony forming units in plate-based tests, Ct values in PCR-based analysis, or optical density in colorimetric analyses. When comparing test characteristics across studies, critical interpretation of the results is required as study designs (e.g. definitions, reference tests, thresholds used), study populations (animal and pathogen populations), and methods to calculate confidence intervals may vary and make estimates for scientific criteria study-specific. The summary of POC tests provided in the online Supplementary Table S1 should be read with those cautionary notes in mind. For on-farm application, predictive values are more important than sensitivity and specificity, but they are rarely reported because they are dependent on pathogen prevalence and hence population-specific. A high positive predictive value means that no unnecessary treatment is given ("treat as little as possible"), which achieves the aim of reducing AMU, whereas a high negative predictive value means that treatment is only withheld when cows truly do not need it ("treat as much as needed"), so that cow welfare is not compromised.

Level of differentiation

A crucial question is how POC test results inform decision making. Several plate-based assays have been developed to identify mastitis pathogens to species or genus level (see section

What do we have?), but the rationale for use of selective treatment for mild to moderate CM is largely based on differentiation of gram-positive versus other forms of mastitis. Hence, one could argue that a POC test only needs to give information about presence of gram-positive organism. In addition, it needs to distinguish between contamination and IMI. If genus or species level information influences treatment decisions, however, for example by differentiating *Streptococcus* spp. from *Staphylococcus aureus* or *S. aureus* from non-aureus Staphylococci, this is relevant at POC level. In some countries, narrow spectrum penicillins are the 1st line of treatment for CM, in which case differentiation of penicillin-susceptible and penicillin-resistant isolates may be useful, particularly for *S. aureus* (Barkema *et al.* 2006). Some POC tests now include antimicrobial susceptibility testing (Jones *et al.* 2019). When susceptibility testing is conducted at sample level rather than isolate level, without the ability to differentiate between pure cultures, mixed cultures and contamination, the value of such testing to inform treatment decisions is unknown. In addition, its value may differ between farming systems and regions. For example, predominance of *Strep. uberis* without presence of *Escherichia coli* as a major cause of CM is uniquely associated with the pasture-based system in New Zealand and predictive values from that system may not translate to other dairy-production systems or regions (Bates *et al.* 2020). A recent study suggested a 24% reduction of AMU could be achieved when using a POC test with susceptibility testing, yet no change in milk withhold was observed, which is surprising as milk of treated cows should be withheld (Bates *et al.* 2020).

Societal criteria

Like convenience criteria (cost versus ease of use) or scientific criteria (sensitivity versus specificity), societal drivers for POC test use may be at odds with each other. For animal welfare reasons, a "security blanket" of broad-spectrum antimicrobials for all CM cases may be preferred, but this does not sit well with calls to reduce and refine AMU. From an economic point of view, the value of diagnostic tests is situation-specific. Like predictive values, the value of information depends on pathogen prevalence. If most CM is caused by a single species, the cost of testing may not be outweighed by its financial benefits (Cha *et al.* 2016). This line of reasoning does not account for the potential introduction of AMU quota, which, like milk quota, extend the impact of decisions at individual level to other cows in the herd.

Societal concerns may also exist around work health and safety. On-farm bacteriology by non-experienced people is forbidden is some countries because of concerns about propagating pathogens, including hazard group 2 pathogens which can infect and kill people. Several major mastitis pathogens belong to this category, including *E. coli*, *Klebsiella* spp., *S. aureus*, and *Streptococcus agalactiae* (Zadoks *et al.* 2011). In addition to concerns about hazardous waste, environmental implications of POC testing may need to be considered. Ideally, POC tests would use materials that are bio-degradable or come from renewable sources.

What do we have?

We currently have POC tests that detect mastitis (inflammation) or pathogen presence (Adkins & Middleton, 2018). Inflammation is an indirect measure of infection whereas pathogen detection is a direct measure of infection, with the caveat that pathogen detection without evidence of inflammation may not be sufficient evidence for IMI nor adequate justification for AMU (Nyman *et al.* 2016). All currently available on-farm diagnostics for pathogen identification are culture based (online Supplementary Table S1, whilst culture-independent methods are available off-farm or underdevelopment for on-farm use.

Culture-dependent pathogen information

Several formats are available for culture-based POC tests, including petrifilm, agar plates, and tube-based systems. Some identify bacteria as gram-positive, gram-negative, or absent, e.g. MastDecide, Petrifilm, Point-of-Cow, and VétoSlide. Others identify bacteria to genus and species-level, e.g. VétoRapid, Minnesota Easy Culture Tri-plate, Accumast, SSGN plate, Hardy Diagnosis Triplate, Micromast, Dip-Slide Mastitis, Selma and Selma Plus. Some assays include antibiotic susceptibility testing, e.g. Mastatest, Point-of-Cow and Speed Mam colour. All tests are more reliable when used for diagnosis of broad categories (growth, gram-positive, gram-negative) than at genus or species level (Lago & Godden, 2018). Broad categories may suffice to inform treatment decisions but for culling decisions, more detail or accuracy can be desirable. For such decisions, however, TAT is often less critical, and laboratory-based testing may be more appropriate than on-farm testing.

Reported sensitivities for gram-positive bacteria range from 58.6% (MastDecide; Leimbach & Krömker, 2018) to 98% (Minnesota easy culture system Bi-plate; McCarron *et al.* 2009a), and specificities from 48% (Petrifilm; MacDonald, 2011) to 97% (MastDecide). The inherent trade-off between sensitivity and specificity tends to limit accuracy, although exceptions exist, e.g. for *E. coli* (online Supplementary Table S1). Low pathogen prevalence in POC test evaluation studies will result in wide confidence intervals around point estimates for test characteristics. Interestingly, *Strep. agalactiae* is a major pathogen in many emerging dairy industries but almost no information is available on accuracy of POC tests for this organism (online Supplementary Table S1). The market that is currently targeted for POC testing presumes a certain standard of farm management, which would generally be associated with successful control of *Strep. agalactiae*. Accuracy also depends on observer skills. For example, the ability to detect *S. aureus* based on haemolysis depends on the population under investigation and the experience of the reader (McCarron *et al.* 2009b). Petrifilm or agar-based tests tend to involve relatively cheap equipment and reagents. However, they require considerable user training, as well as sufficiently high throughput to maintain user skills. Agar-based tests often allow for easy visual identification of sample contamination, which is important to monitor sample quality. Unfortunately, not all user manuals provide guidance on the distinction between positive and contaminated samples, or its importance. In recent market introductions, such as Point-of-Cow and Mastatest (Jones *et al.* 2019), consumables and equipment are more sophisticated and more expensive to increase ease of use. Although the test is run on-farm, data interpretation may happen off-farm, using cloud-based computing systems. Issues around data confidentiality and data ownership in such systems may need further consideration, especially if test data is combined with data at cow or farm level. Culture-based tests all have similar TAT of up to 24 hrs. Although this is fast enough to inform decision making, farmers would prefer less delay with turn-around from one milking to the next (Griffioen *et al.* 2016).

Culture independent pathogen information

Commercially available culture-independent pathogen detection systems are currently based on polymerase chain reaction (PCR), a method to selective amplify target DNA of interest. Targets may be genus specific, species specific, or indicative of AMR, as in the case of the *bla*Z gene encoding penicillin resistance in *Staph. aureus*. DNA amplification is possible even when bacteria are non-viable or in a viable but non-culturable state, which enables

pathogen detection in culture-negative samples. This is probably more relevant for samples that are shipped to a laboratory than for POC testing and use of PCR is largely limited to professional laboratories. Whether detection of bacterial DNA in culture negative samples provides justification for AMU is an open question (Nyman *et al.* 2016). Growing recognition of the existence of a mammary microbiota, and descriptions of mastitis as a "dysbiosis", suggest that presence of DNA in low quantities may be normal. Studies on associations between treatment and cure have historically been culture based. If or how treatment impacts on the outcome of CM cases with PCR-positive, culture-negative results is untested.

To combine the scientific characteristics of DNA-based testing with the convenience characteristics of on-farm testing, loop-mediated isothermal amplification (LAMP) of DNA is explored. This test uses different chemistry than PCR, requires less sample preparation, and is less sensitive to inhibitory substances present in biological samples (Bosward *et al.* 2016). It can be configured as a pregnancy test-like lateral flow device, which could be a rapid and cost-effective on-farm diagnostic (Cornelissen *et al.* 2016). LAMP has received attention in the scientific literature as a means to make molecular diagnostics feasible and affordable in low resource settings. Its implementation as on-farm POC test for CM is hampered by heterogeneity of mastitis pathogens and resistance determinants and the limited ability for multiplex testing. The latter may be addressed through developments in microfluids (see Future developments).

Future developments

Technological development progresses extremely quickly, demonstrated by revolutionary changes in computing, mobile phone technology, and DNA-sequencing over recent decades. The biology of CM in dairy cattle has not changed as much over the same period, although developments in genetics and husbandry have led to significant shifts in milk production and predominant pathogen populations in developed countries. Societal attitudes towards AMU have changed more recently and influence availability and use of antimicrobials for CM treatment. Possibly most resistant to change is human behaviour, which drives the need for as well as the development and uptake of diagnostics. In this section, we discuss opportunities to harness the power of the technological revolution, the data revolution, and the people that milk or manage cows (Figure 1).

Harnessing the power of technology

Advances in microfluidics allow development of technologies that can be incorporated into automatic monitoring systems and portable devices for sensitive and rapid mastitis diagnostics (Viguier *et al.* 2009). After somatic cell count, perhaps biomarkers such as acute phase proteins (APPs) are the most widely studied inflammatory biomarkers in milk. Haptoglobin (Hp) and milk-amyloid A (MAA) are especially prominent members of this group in dairy cattle and both are synthesised locally in mammary tissue. To inform treatment decisions, biomarker patterns would need to be pathogen-specific. Whilst there is some evidence to support this approach, which could be enhanced by multiplex assays linked to machine learning technology (see next section), it is not always clear whether APP profiles reflect severity of inflammation or causative agents and concerns exist about sensitivity and specificity (Pyörälä *et al.* 2011). Small inflammatory mediators such as cytokines and chemokines have shown significant promise with proteomic platforms differentiating the host response to different pathogens (Kusebauch *et al.* 2018), and progress is being made in the development of biosensors for detecting such biomarkers (reviewed by Martins *et al.* 2019). Increasingly, the

line between biomarker detection and pathogen detection is blurred, as culture-free pathogen detection becomes feasible on microfluidic devices popularly known as “lab on a chip”. One of the key challenges in the development of such devices is the need to detect multiple targets. We are not aware of developments aimed at differentiating gram-positive and other causes of mastitis, although identification of a few major gram-positive mastitis pathogens might suffice to support on-farm treatment decisions. If so, paper-based multiplex LAMP assays may provide a low-cost option that is fast, microbiologically safe (no pathogen amplification, paper can be burned after use) and environmentally sustainable (Reboud *et al.* 2019).

Harnessing the power of data

Technological developments should be accompanied by developments in data analysis to maximise the benefit from increasingly sophisticated biomarker or pathogen detection systems.

Machine learning methods became popular for many applications in which predictive performance is the main aim. This type of narrow artificial intelligence has been applied to mastitis diagnosis, focusing on real-time detection from milking data. Artificial neural networks (ANN) and tree based models perform best in this context (Ebrahimie *et al.* 2018; Ebrahimi *et al.* 2019), linking parameters such as milk yield, electrical conductivity, and lactose level to SCM. This black-box type of model is optimised for prediction at the cost of interpretability, which is a good trade-off for real-time mastitis diagnosis. Adding more layers of weights to ANNs gives rise to the “deep learning” models that are successful in many fields. Dhoble *et al*. (2019) recommends neural networks also for label-free flow cytometry data to routinely screen for mastitis.

By contrast, simple classification tree models are easy to interpret, and cut-off values calculated from them are easily applicable in POC tests such as lateral flow immunoassays. More complex models (random forests, gradient boosted trees) gain predictive accuracy at the cost of interpretability (Ebrahimi *et al.* 2019). Like neural networks, complex tree models are mostly black boxes to the user. Machine learning methods are more flexible than classic multivariate statistical methods, requiring fewer assumptions about the data generating mechanisms and independence of various measurements. They can combine multiple data types into a single prediction model, making them useful for mastitis diagnosis based on a combination of demographic, epidemiological, milking, flow cytometry, and biomarker data. However, the lack of human oversight in the model specification process is also the drawback of many machine learning models. Biases in the data on which these models are trained tend to get reinforced in the model fitting process, reducing the applicability of the model to the wider animal/farm population. Training data sets should therefore be representative of the target population, and also relatively large to avoid overfitting.

In the era of cheap sensors, the goal will be to develop rapid (semi-)automated diagnostic systems that apply artificial intelligence to real-time data streams (e.g. from automated milking) and biomarker information to distinguish between gram-positive and gram-negative pathogens. Differences in baseline parameters between farms will pose a challenge, which can be overcome by either standardising the machine learning models, or retraining them for use on new farms (Ebrahimi *et al.* 2019). Ideally, data from diagnostic systems would be combined with prognostic factors, such as duration of infection (SCC and CM history), parity, as well as measures of cow value (genetic merit and lactational performance) to weigh the probability of treatment success against the value of the individual animal. Studies on prognostic indicators

are limited but suggest the importance of similar factors across multiple gram-positive organisms (Barkema *et al.* 2006, Samson *et al.* 2016).

Harnessing the power of people

Scientific research has generated sufficient knowledge to support targeted treatment of non-severe CM based on pathogen identification and cow factors. However, this knowledge is hardly implemented and farmers frequently experience "insecurity" and "uncertainty" about mastitis treatment (Swinkels *et al.* 2015). Such feelings may motivate farmers to seek social approval and emulate peer behaviour, for example by extending treatment where increased AMU is perceived to be "better" (Swinkels *et al.* 2015). Ideally, the same social phenomenon would be harnessed to reduce AMU, e.g. by training "champions" of antimicrobial stewardship and using peer networks to spread prudent AMU. Farmer-led approaches rather than traditional passive knowledge transfer methods may be the best way to motivate change in AMU or POC test use (Bard *et al.* 2017). In addition, communication approaches like motivational interviewing, which are designed to facilitate clients' internal motivation to change may improve uptake of veterinarian advice (Bard *et al.* 2019; Svensson *et al.* 2019). Demographic factors and affective attributes, such as a veterinarian's age, respectfulness and dominance, or a farmer's education level also influence farmers' satisfaction and willingness to adopt veterinary advice (Ritter *et al.* 2019). Such insight could be implemented to promote POC tests uptake and targeted treatment approaches.

Farmers and veterinarians are part of food production and health care systems that may provide barriers or incentives for behaviour change. Perceived barriers at farm level include lack of time, economic investment with no financial short-term benefits and labour shortages (Ritter *et al.* 2017). Drivers for uptake may include improvement of cows' welfare and farm profitability, long-term job satisfaction, reputational benefits in relation to consumer demands, social recognition and pride, and the desire to conform to perceived standards of "being a good farmer" (Swinkels *et al.* 2015). In some countries AMU quota have been implemented successfully or recommended to reduce AMU (Bos *et al.* 2015; O'Neill, 2016). Benchmarking of individual AMU against average AMU of peers or at national level can provide a sense of being part of a nationwide reduction campaign and may encourage POC test uptake, as farmers are more inclined to assume their responsibility as a part of joint effort (Ritter *et al.* 2017). It would be naïve, however, to ignore the role of commercial drivers in uptake of selective treatment to reduce AMU. Many veterinarians generate income from sales of antimicrobials, and this may serve as a disincentive for promotion of targeted treatment. In many countries, antimicrobial use and sales are not under veterinary control, which makes the issue even more complicated. Contracts and demands from milk processors and retailers may override preferences or recommendations from farmers and veterinarians. The balance between "stick" (forced reduction in AMU) and "carrot" (financial and reputational benefits from reduction in AMU) will differ between countries and production systems and change over time.

Final thoughts

The 3Rs of Reduction, Refinement and Replacement are well known in the context of experimental animal research. The same concepts could be applied to antimicrobial treatment of mastitis. Reduction can be achieved if AMU for non-severe CM is targeted to gram-positive IMI only. As reviewed here, this may require further improvements in convenience characteristics and scientific criteria of POC tests and changes in knowledge, attitudes and

behaviours among veterinarians and dairy farmers. Behaviour change should also include refinement, whereby we should abstain from the use of HP-CIA in dairy cattle (Turner *et al.* 2018). Professionals in the dairy industry, processors, retailers, pharmaceutical companies and regulators all have a role to play in reduction and refinement as they buy milk, or produce, approve and market antimicrobial products. Ideally, refinement of herd management practices would reduce the incidence of mastitis and the need for treatment. The concept of replacement is used in dry cow treatment, where internal teat sealants increasingly replace antimicrobials as tools for infection prevention. To some extent, anti-inflammatory and supportive therapy can act as replacements in treatment of lactational CM. They are not causative in removing the infectious agent but contribute to a successful host response. Alternatives that target mastitis pathogens, such as biocins and phage therapy, require further development (reviewed by Angelopoulou *et al.* 2019).

Regardless of the need for further research on replacement, we already have the tools and the knowledge to reduce and refine the use of antimicrobials in treatment of lactational mastitis. The onus is on the veterinary profession, the farming industry, and associated industries and regulators to decide how best to incentivise and implement them.

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Figure legends:

Figure 1:

Point-of-care tests for bovine clinical mastitis. What do we have and what do we need.

Table legends:

Supplemental Table S1:

What do we have as point-of-care (POC) tests for bovine clinical mastitis? Characteristics summary of point-of-care tests available on the market for pathogen identification.

Figure 1:

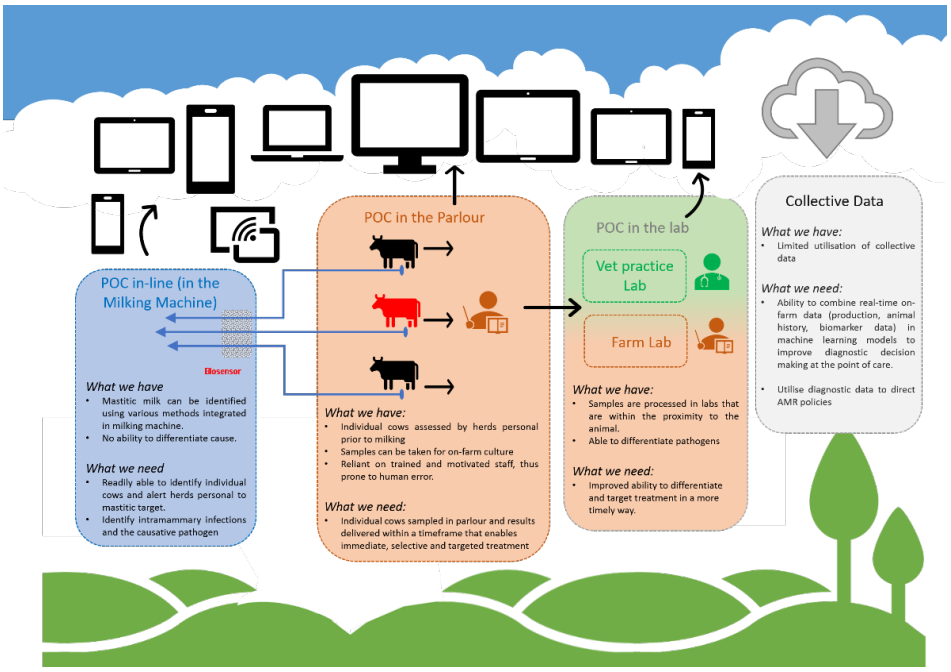

Table S1. What do we have as point-of-care (POC) tests for bovine clinical mastitis? Characteristics summary of point-of-care tests available on the market for pathogen identification. (Table 1 near end section "3.1 Culture-dependent pathogen information")

Test	Number/type of selective media	Pathogens identified by test	Turn-around time	Price per test[1]	Lab or farm-based validation	Sensitivity (%)	Specificity (%)	Accuracy (%)	References[2]
VétoSlide	Biplate: Gram-ve Gram+ve	No bacterial growth Gram+ve Gram-ve *E. coli*	24-48h	~ £6	Lab	82.0[a] 83.3[b] 84.3[c]	76.8[a] 94.3[b] 97.5[c]	79.2[a] 89.2[b] 92.3[c]	Malcata *et al.* submitted
VétoRapid	Triplate: Gram-ve *Staph.* *Strep. & enterococci*	No bacterial growth *E. coli* *Klebsiella spp* *Staph. aureus* Non-aureus staph. (NAS) *Strep. uberis Enterococcus spp.* *Strep. dysgalactiae* *Strep. agalactiae*	24-48h	~ £13	Lab	91.3[a] NA[b] 66.7[c] 83.3[d] 92.3[e]	77.7[a] NA[b] 92.5[c] 93.5[d] 94.5[e]	82.4[a] NA[b] 86.8[c] 92.6[d] 94.1[e]	Viora *et al.* 2014
						88.5[a] 83.3[b] 86.3[c] 100[d] 90.5[e]	58a 88.6b 92.4c 70.7d 82.6[e]	72.3a 86.2b 90.0c 72.3d 83.8[e]	Malcata *et al.* submitted
Minnesota Easy Culture-Biplate	Biplate: Gram+ve Gram-ve	No bacterial growth Gram +ve Gram -ve *Staph. aureus*	18-24h	~ £1.55	Farm	78.4[a] 73.1[b]	82.5[a] 86.5[b]	81.0[a] 83.0[b]	Lago, 2009
					Lab	97.9[a]	67.5[a]	NA[a]	McCarron *et al.* 2009a
						80-85[a] 58-72[b] 50-69[c] 58-63[d] 3[e]	79-87[a] 92-97[b] 93-97[c] 92-96[d] 99[e]	81-84[a] 89-90[b] 91-94[c] 91[d] 82[e]	Royster *et al.* 2014
Minnesota Easy Culture-Triplate	Triplate: Gram +ve Gram –ve *Strep. & Strep-* like	No bacterial growth Gram +ve Gram -ve *Staph. aureus* *Staph.* spp. *Strep.* spp.	18-24h		Lab	78[a] 98[d] Different readers: 43.2-59.1[d]	78[a] 82[d] Different readers: 93.8-95.9[d]	NA	McCarron *et al.* 2009b
						80-86[a] 60-78[b] 50-69[c] 52-78[d] 5-11[e]	76-93[a] 96-97[b] 93-97[c] 92-98[d] 97-98[e]	83[a] 90-93[b] 91-94[c] 90-93[d] 29-57[e]	Royster *et al.* 2014
						60.0a 55.6b	82.9a 93.9b	81.3a 80.2b	Ferreira *et al.* 2018

Table S1. *(Cont.)*

Test	Number/type of selective media	Pathogens identified by test	Turn-around time	Price per test[1]	Lab or farm-based validation	Sensitivity (%)	Specificity (%)	Accuracy (%)	References[2]
Petrifilm (Aerobic Count, Coliform Count, Staph Express Disk)	One	Using Aerobic Count & Coliform Count together: No bacterial growth Gram +ve Gram –ve *Staph. aureus* (Staph Express Disk)	24h + 3h with STX Disk 24h + 3h with STX Disk 24h	~ £1.50-3.50	Lab	93.8[a] 94[a] Experienced reader: 97.4[d] 92.1[d] (STX Disk)	70.1[a] 70[a] Experienced reader: 76.1[d] 93.1[d] (STX Disk)	NA NA	McCarron *et al*. 2009a McCarron *et al*. 2009b
					Farm	64[a]	48[a]	NA	MacDonald, 2011
					Farm (by trained researcher)	85.2[a] 89.9[b] 88[d]	75.4[a] 88.4[b] 66[d]	NA	Mansion-de Vries *et al.* 2014
MastDecide	Two: Growth Gram +ve	No bacterial growth Gram +ve Gram -ve/coliform	12-14h	~ £8.50	Lab	58.6-85.3[a] 58.3-72.2[b]	81.5-97[a] 78.1-94[b]	58.6-85.3[a] 58.3-72.2[b]	Leimbach & Krömker, 2018
AccuMast	Triplate: Gram -ve *Streptococci/ Enterococci* *Staphylococci*	No bacterial growth Gram -ve *E. coli* *Klebsiella*/*Enterobacter* *Strep. uberis* *Enterococcus* spp. *Lactococcus* spp. *Staph*. spp. *Staph. aure*us	16-20h	~ £5.50	Farm (by trained researcher)	81.6[b] 75.0[c] 100[d]	98.9[b] 97.9[c] 99.8[d]	96.4[b] 95.7[c] 99.8[d]	Ganda *et al*. 2016;
					Lab	100[c]	95.5[c]	96.8[c]	Ferreira *et al*. 2018
SSGN Plate	Quad plate: Growth Gram -ve *Streptococci* *Staphylococci*	No bacterial growth *Staph.* spp. *Staph. aureus* *Strep.* spp. *Strep.. agalactiae* Gram -ve *E. coli*/coliforms	24-48h	~ £2.50	Lab	73.3[c]	92.4[c]	90.3[c]	Ferreira *et al*. 2018
Mastatest	One plate containing 24 wells	No bacteria *Strep. uberis* *Staph. aureus* *Staph.* spp. Gram -ve	22h	NA	Lab	80[a] 77[b] 85[d] 88[e]	94[a] 100[b] 96[d] 80[e]	NA	Jones *et al.* 2019

Table S1. *(Cont.)*

Test	Number/type of selective media	Pathogens identified by test	Turn-around time	Price per test[1]	Lab or farm-based validation	Sensitivity (%)	Specificity (%)	Accuracy (%)	References[2]
NeoFilm	Two: Growth Gram -ve	No bacterial growth Gram +ve Gram -ve	NA	NA	NA	NA	NA	NA	Lago & Godden, 2018
Point-of-cow	One cassette: Gram +ve Gram -ve	No bacterial growth Gram +ve Gram -ve	6-16h	~ £7.00	NV	NV	NV	NV	Fluimedix, 2017
MicroMast	Triplate: Gram -ve Streptococci Blood sheep agar non selective	*E. coli* *Klebsiella* spp *Staph. aureus* Non-aureus staph. (NAS) *Strep. uberis Enterococcus* spp. *Strep. dysgalactiae* *Strep. agalactiae*	24h	~£5	NV	NV	NV	NV	Prášek, 2017
Selma	Triplate: Growth Gram –ve *Staphylococci/ Enterococci*	No bacterial growth *Staph*. spp. *Staph*. aureus *Strep*. spp. Gram -ve	18-48h	~£2.80	NV	NV	NV	NV	SVA, 2019
Dip-slide Mastitis	Three: Growth Gram +ve Gram -ve	*Strep. uberis* *Staph. agalactiae* *Staph. aureus* *Pseudomonas* *Klebsiella* spp *E. coli* *Proteus mirabilis*	18-48h	NA	NV	NV	NV	NV	Axonlab, 2019
Speed Mam Color	One mini culture plate	*Staph*. spp *Strep* spp *Strep. uberis* *E. coli* *Enterobacteriaceae* *Enterococcus* spp. *Mycoplasma* spp *Pseudomonas*	24-48h	NA	NV	NV	NV	NV	Manner, 2001

[a]Gram+ve, [b]Gram-ve/coliform, [c]*E. coli*, [d]*S. aureus*, [e]*S. uberis*. NA: Information not available, NV: not validated

[1]Price excludes any initial set-up costs; [2]Reference detailed information provided in the supplementary information

References of the supplementary information Table S1

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