Diagnostic information for mastitis treatments on dairy farms

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Diagnostic information for mastitis treatments on dairy farms

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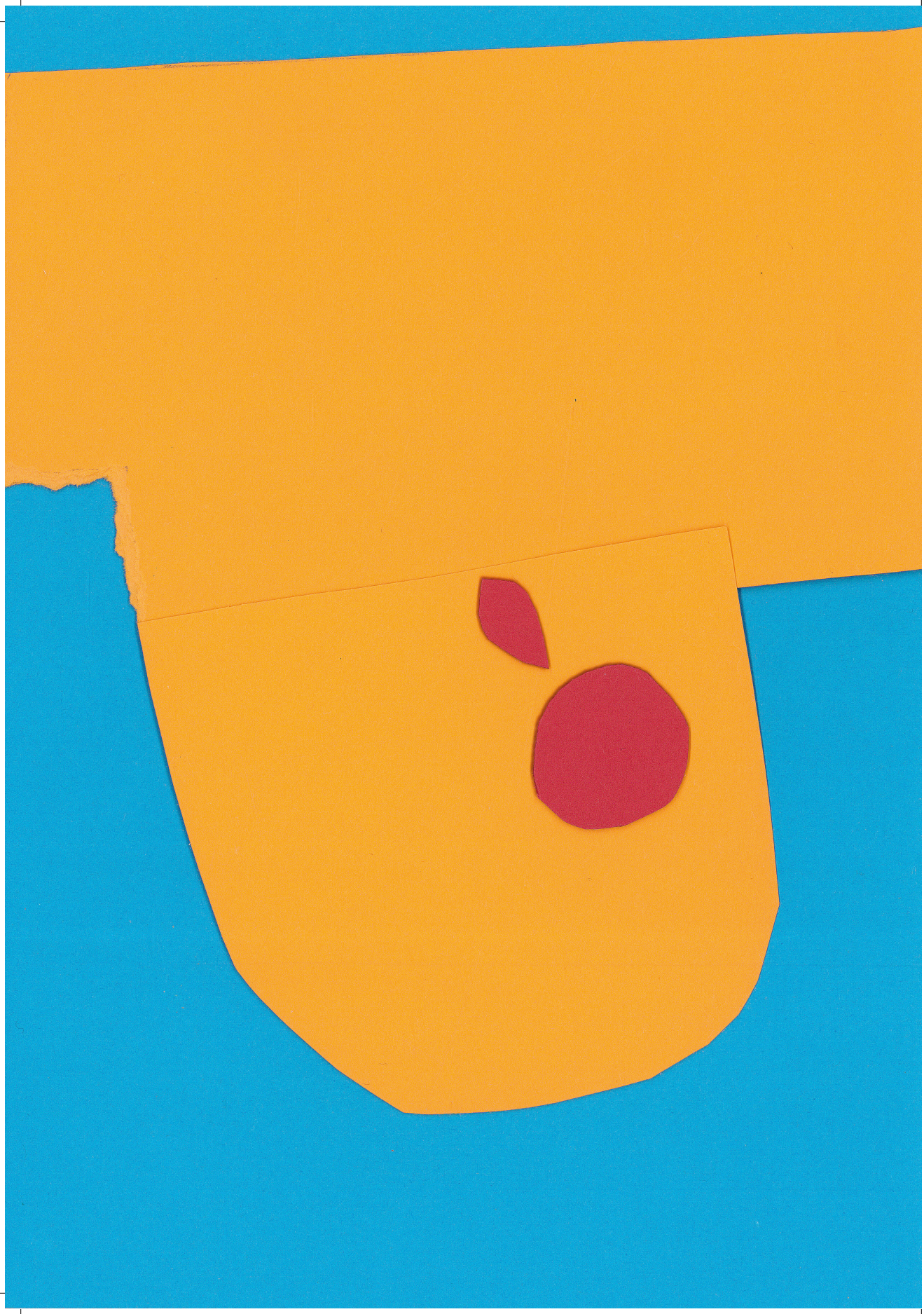

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General introduction

Within the *in vitro* diagnostics market, growth is mainly foreseen in point-of-care (POC) tests and molecular tests (Abel, 2015; Huckle, 2015). Testing at the POC means that the test is performed near the patient as opposed to testing in a laboratory. In human medicine, reasons for POC testing are that immediate results can modify or optimize treatment of patients without a delay due to sample transfer to a laboratory. Also in veterinary medicine POC tests are increasingly used. Two main reasons for that are the potential decreased costs of making a diagnosis, and the reduced time between sample collection and diagnosis which facilitate treatment decision (Busin et al., 2016). Therefore, using POC tests can support timely management of diseased animals and can help to target treatment decisions.

All sorts of test results and other data are increasingly used on dairy farms, together captured in the concept 'precision dairy farming'. Precision dairy farming uses technologies to monitor physiological, behavioral, and production indicators of individual cows. The interest in such technologies comes from increasing herd sizes and growing importance of efficient farming. Goal of incorporating technologies on dairy farms is to maximize animal performance, and thus to detect diseased cows and production problems early, in order to monitor herd level health and to minimize the use of medication through preventive health measures. Examples of these technologies are sensors that measure body condition, pH of the rumen, urea, or activity meters for estrus detection (Rutten, 2017). However, the first application of the use of sensors on farms was for the detection of clinical mastitis with electrical conductivity sensors (Nielen et al., 1992; Hogeveen et al., 2010).

Mastitis

Mastitis, inflammation of the mammary gland, affects many species (Barlow, 2011) and is most often caused by an intramammary infection (IMI) with bacteria, but also yeasts, fungi, and algae can play a role (Watts, 1988; Hillerton and Berry, 2005). In dairy cows, mastitis can be differentiated in clinical and subclinical mastitis. Clinical mastitis is characterized by visible signs, like cloths or flakes in the milk, or redness or swelling in the udder. Also systemic symptoms might be present, like fever or loss of appetite. Subclinical mastitis has no visible signs, but is defined by the number of somatic cells (SCC) present in milk as determined with further diagnostics. Somatic cells are a measure of inflammation, and comprise mainly macrophages, neutrophils,

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epithelial cells, and mononuclear cells (Pyörälä, 2003). The cut-off values to assess whether SCC is high or low, and thus whether subclinical mastitis is present, differ in literature (Berry and Meaney, 2006; Nyman et al., 2016; Hawkins, 2019). Generally, a cow average composite SCC (CSCC) of ≥ 200,000 cells/mL is considered high (Dohoo and Leslie, 1991; Schukken et al., 2003). However, in the Netherlands a CSCC ≥ 250,000 cells/mL is considered high for multiparous cows whereas that is a CSCC ≥ 150,000 cells/mL for primiparous cows (Vanholder and Melchior, 2012; CRV, 2019).

Mastitis is the most prevalent and costly disease on dairy farms, and is responsible for almost 70% of the antimicrobial usage at farms (Pol and Ruegg, 2007; Hill et al., 2009; Kuipers et al., 2015). Most of the cows with clinical mastitis are treated with antimicrobials (Vaarst et al., 2002; Hill et al., 2009; Santman-Berends et al., 2016). Subclinical mastitis is hardly treated during lactation, but is frequently treated at drying off (cessation of milking roughly two months before expected calving date) (Halasa et al., 2009). Dutch farmers observed and recorded an incidence rate of clinical mastitis of 33.4 per 100 cows per year (Santman-Berends et al., 2015), although high variation is reported between studies among different countries. For example on large US farms an incidence rate of 65.9 per 100 cows per year was reported (Evink and Endres, 2017), and on Canadian farms an incidence rate of 23.0 per 100 cows per year (Olde Riekerink et al., 2008). For subclinical mastitis a prevalence of 22.2% was recorded in the Netherlands in 2009 (Lam et al., 2013), which decreased to 15.8% in 2013 (Santman-Berends et al., 2016).

Mastitis treatment decisions

For each cow with mastitis, farmers need to decide whether or not to treat it with antimicrobials or to use another approach. For animal welfare reasons, severe cases obviously are treated as good as possible, but apart from that attention has to be given to prevention of new IMI. Due to the infectious nature of mastitis, treatment of infected animals can prevent transmission of pathogens to noninfected animals, next to, for example, segregation or culling (Barkema et al., 2006).

Decision-making is a complex process, and farmers often question themselves whether and how to treat a cow with mastitis. To decide on mastitis treatment, many aspects are considered by farmers. Four levels of decisions have been described that influence

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mastitis treatments (Vaarst et al., 2002). The first level is the disease level, which is based on clinical signs and is currently most often used by farmers to decide on treatment of clinical mastitis (Pinzón-Sánchez et al., 2011). The second level is the cow level, which adds cow information like parity and days in milk to the decision-making process. The third level is the herd level, which additionally includes the herd situation like the number of cows, the milk quota in place, or societal related factors. The fourth level is the level of alternatives, which comprise alternative options for antimicrobial treatment that are available to farmers, like blinding a teat. Together, these levels result in the decision made by the farmer if and how a cow will be treated. The first level is most important with respect to treatment decisions for clinical mastitis, especially for more severe cases. The other levels are relatively more important with respect to treatment decisions for subclinical mastitis or less severe clinical mastitis cases. Furthermore, at the third level, not only the question needs to be asked whether or not an antimicrobial treatment is regarded as necessary. Also, the possible consequences of antimicrobial treatments in animals should be considered, like potential antimicrobial resistance development of bacteria. These two considerations on antimicrobial treatments in animals have gained more attention over the last decades.

Legislation on antimicrobial use in the Netherlands

The awareness of increasing antimicrobial resistance in bacteria in both livestock and animal products, and the contribution of animal antimicrobial treatments on its prevalence and spread, has resulted in increased consumers concerns about the origin and quality of their food (Bager et al., 2000). As a reaction, big retail companies formulated a position on treatment of animals in their mission statements. To reduce the development of antimicrobial resistance in the Netherlands, the Taskforce Antibiotic Resistance in Animal Husbandry was set-up, formed by representatives of the animal sectors. The Taskforce aimed at more responsible use of antimicrobials and for that each husbandry sector formulated action plans (Speksnijder et al., 2015). For the dairy cattle sector this was done by the taskforce on antimicrobial usage in cattle. In 2010, the Netherlands Veterinary Medicines Institute (SDa) was founded, and set out to collect data on antimicrobial prescription and usage on all Dutch farms (approximately 50,000), and to define targets for antimicrobial usage for each livestock species (Speksnijder et al., 2015). In 2011, the Dutch government banned preventive use of antimicrobials,

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which was one of the major steps in reducing antimicrobial usage in the Netherlands (Speksnijder et al., 2015).

Point-of-care mastitis diagnostics

Because preventive use of antimicrobials is no longer allowed in the Netherlands, the practice of treating all cows at drying off, known as blanket dry-cow treatment, was no longer possible. Therefore, alternative approaches were needed to treat and control IMI during the dry period on dairy farms. One of the actions was to incorporate diagnostic test results in the decision-making process for dry-cow treatment. For that, CSCC levels are used most often (Scherpenzeel et al., 2014). Based on the CSCC levels, a farmer can decide to apply an antimicrobial treatment curatively to those cases likely with an IMI at drying off.

Also during lactation there might be a need for tests to support mastitis treatment decisions, for example tests that help to distinguish mastitis cases that are likely to respond to an antimicrobial treatment from cases that might not benefit, or tests that help to decide on the type of antimicrobial to be used. These considerations on treatment are captured in the term 'prudent antimicrobial use', which comprises both the quantitative usage of antimicrobials, and the efficacy of the antimicrobials used. Thus, as little as possible and as much as needed. To use antimicrobials prudently, antimicrobials should only be applied to mastitis cases with an IMI. Additionally, within the cases with an IMI, one should distinguish those cases that are likely to respond to the antimicrobials chosen from those that are unlikely to respond. For both these aspects, diagnostic tests can provide useful information.

There is a need for detection of clinical mastitis with the introduction of automatic milking systems. With this system, the visual detection of clinical signs of abnormal milk lacks during milking. As a consequence, various tests have been developed over time to support farmers in diagnosing mastitis at an early stage, with for example, in-line tests to determine SCC, lactate dehydrogenase concentration, electrical conductivity, and the chemical composition of milk (Milner et al., 1997; Friggens et al., 2007; Hiss et al., 2007; Fosgate et al., 2013; Nyman et al., 2016) (Table 1). Although these tests often have been developed for automatic milking systems, many of them are also used on farms with conventional milking systems.

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Table 1. Overview of POC tests available to indicate mastitis on dairy farms

Test	Test result	Remarks	Characteristics[1]
CMT (Sargeant et al., 2001) (Dingwell et al., 2003) (Vanholder and Melchior, 2012)	Indirect indication of SCC	Weak reactions (SCC 200,000 - 400,000 cells/mL) can be missed while these quarters can be infected. Subjective judgment.	Se 0.20 – 0.95; 0.75 – 0.87[2]; 0.28 – 0.98[3] Sp 0.50 – 0.80; 0.26 – 0.50[2]; 0.40 – 0.89[3]
PortaSCC (Amaral and Ruegg, 2011)	Indirect indication of SCC	Readout with color chart or digital. Numerical result possible.	Accuracy 0.88[4]
RT10 Dairy Tuner	Indirect indication of SCC	If high SCC, then also indication of causative pathogen based on SCC.	No literature available
LDH concentration (Hiss et al., 2007)	Indication of LDH concentration	Readout with color chart. Usability for mastitis detection is under discussion.	Se 0.81 – 0.93 Sp 0.81
Conductivity		Conductivity has high agreement with SCC. Sensitivity for mastitis detection is low. In-line measurement is an opportunity to collect data each milking.	
Hand-held (Fosgate et al., 2013)	Electrical conductivity		Se 0.30 – 0.87 Sp 0.26 – 0.90
In-line (Ruegg and Reinemann, 2002)	Electrical conductivity		Se 0.32 – 1.00 Sp 0.69 – 1.00
Sensors (Hogeveen et al., 2010)	Widely varying, for example cow activity or color of milk	Mastitis detection might be possible if sensors are combined. Opportunity for continuous data collection.	Se 0.47 – 1.00 Sp 0.69 – 1.00

CMT = California Mastitis Test; SCC = somatic cell count; LDH = lactate dehydrogenase
[1] Average sensitivity (Se) and specificity (Sp), unless specified otherwise
[2] At the end of lactation
[3] At the beginning of lactation
[4] Accuracy is the total of results in agreement with the reference test. In this case between PortaSCC and SCC

The oldest tests known are tests to measure SCC. Such tests are available to many farmers worldwide and know many applications. For example, SCC is determined in bulk milk by the milking plant and used as a measure of milk quality. Also at the cow level, CSCC is used as a management tool to control mastitis, in the form of test-day records (Vanholder and Melchior, 2012; CRV, 2019). For that, cows are sampled with regular intervals during lactation, often of 4 to 6 weeks, resulting in CSCC lactation curves (de Haas et al., 2005). Because it is much easier to collect CSCC data than to culture bacteria, many studies investigated the relation between CSCC and IMI (Suriyasathaporn et al., 2000; Berry and Meaney, 2006; Reksen et al., 2008; van den Borne et al., 2011). CSCC patterns were found to be indicative of specific mastitis pathogens and therefore are useful in management decisions (de Haas et al., 2004). However, CSCC data are not

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specific enough to make treatment decisions, which are often made at the quarter level. The Californian Mastitis Test (CMT) gives an indication on SCC at the quarter level on-farm. The CMT is a rapid and easy to use test, that indicates inflammation by the formation of aggregation between the somatic cells present in the milk and an added detergent. Studies have looked for the association between mastitis causing pathogens and the CMT result. These studies conclude that CMT can be used as a screening method to select individual cows for bacteriological culture (Sargeant et al., 2001; Sandford et al., 2006).

Tests for treatment strategy

A plethora of diagnostic tests is available to assist in using antimicrobials prudently (Pyörälä, 2003; Lam et al., 2009; Viguier et al., 2009). The types of tests range from laboratory tests to simple quick on-farm tests (Koskinen et al., 2009; Mahmmod et al., 2013a; Viora et al., 2014; Ferreira et al., 2018; Leimbach and Krömker, 2018). Laboratory tests, like bacteriological culture, and polymerase chain reactions (PCR) are performed in a central laboratory. Such tests are often highly specific and able to provide information on the pathogen that likely causes the IMI, and whether that pathogen is likely to respond to an antimicrobial treatment (Dohoo et al., 2011b; Gurjar et al., 2012). The downside of laboratory tests is that they are time-consuming, first of all because of the time necessary to transport the milk sample to the laboratory, but also because of the time needed for sample preparation and performing the tests. Of the laboratory tests, bacteriological culture is most often used. Up to recently, there was no consensus on the definition of an IMI, but often the combination of SCC, as a reflection of the inflammatory response, and bacteriological culture, as a reflection of the pathogen present, is used to determine the intramammary status (Dohoo et al., 2011a; Dohoo et al., 2011b). Today, the consensus standard agreed upon by udder health professionals is that the presence of an IMI should be based on three consecutive milk samples cultured, with at least two out of three indicating the same pathogen (Andersen et al., 2010). Due to the impracticality of that approach, hardly ever three milk samples are taken. Thus, bacteriological culture based on a single milk sample is mostly used if information is needed on the IMI, both in field settings as well as in research settings.

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For treatment decisions, bacteriological culture is rarely used due to the long time interval between sampling and result. Nevertheless, to determine mastitis treatment, and to apply antimicrobials prudently, information on the bacteriological cause of an IMI is necessary. Even though testing a single milk sample has limited sensitivity (Se) and specificity (Sp) (Dohoo et al., 2011b), and thus misclassification regarding the real IMI status of the udder from which the milk is collected is possible, it currently is the most practical method to obtain IMI information in field settings. Also in this thesis, bacteriological culture based on a single milk sample was used if information on the bacteriological cause of mastitis is needed. Nowadays, with respect to prudent antimicrobial use in dairy farming, information on the cause of mastitis becomes more important for treatment decisions. Because bacteriological culture is scarcely deployed, quick on-farm tests have been investigated globally for their suitability to gather information on the cause of mastitis (Lago et al., 2011b; MacDonald et al., 2011; Keefe et al., 2013; Mansion-de Vries et al., 2014; Viora et al., 2014).

Objective and outline

Also in the Netherlands, practical diagnostic tools that support farmer and veterinarian to decide on mastitis treatment are hardly used. Nevertheless, farmers often question whether and how to treat cows with mastitis. Thus, there may be a need among dairy farmers for additional information on the cause of an IMI to facilitate treatment decisions and to enhance prudent antimicrobial use.

The aim of this thesis is to determine the added value of diagnostic information on the mastitis treatment strategy of Dutch dairy farmers. The ultimate goal is to contribute to improved mastitis treatments on Dutch dairy farms to enhance prudent antimicrobial use for mastitis.

First, the current practices of Dutch dairy farmers to decide on mastitis treatments were assessed. Additionally, it was determined what the need of dairy farmers was for (new) additional tests in that perspective and the preferred characteristics of such tests if used for treatment decisions. The results of this questionnaire are described in *Chapter 2*.

In literature different tests have been described to be used for mastitis treatment decisions. These tests were screened for the availability in the Netherlands and whether

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they might fit the criteria as indicated by the farmers in *Chapter 2*. Four of these tests were selected and evaluated in the laboratory using mastitis samples routinely sent in for bacteriological culture. Agreement of these four tests with bacteriological culture was determined to detect three diagnostic categories: Gram-positive growth, Gram-negative growth, and no growth. The results of this laboratory evaluation are described in *Chapter 3*.

Furthermore, various test platforms were screened for their suitability to develop a new test to detect mastitis-causing bacteria under Dutch circumstances. Loop-mediated isothermal amplification (LAMP) is one of those platforms. LAMP seems promising for on-site use, as a genetic test that can provide a quick result. LAMP might fulfill the need of the farmers for additional quickly available diagnostic information. Four LAMP assays were developed targeting *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus* spp., and were evaluated with respect to detect their targets. Additionally, an on-site read-out of LAMP was evaluated, being a nucleic immunoassay lateral flow assay (NALFIA) (*Chapter 4*).

Currently, CSCC is used by farmers to decide whether or not to apply antimicrobials at drying off. However, if farmers know whether a pathogen is involved in the IMI and which one, prudent antimicrobial use at drying off might be enhanced. To investigate this, the effect of diagnostic information on farmers' decisions at drying off was determined by comparing the decisions farmers made before IMI information was provided to the decisions made after IMI information was provided to the farmers. These results are described in *Chapter 5*.

Also the effect of diagnostic information on farmers' decisions during lactation were determined. Therefore, two of the four tests evaluated in the laboratory were used by Dutch farmers to determine treatment of clinical and subclinical mastitis during lactation on-farm. The effect of incorporating on-farm tests in the treatment strategy for mastitis were compared to the current treatment strategy farmers apply. Outcomes of interest were antimicrobial use and cure of mastitis. These outcomes had not been described earlier as compared to the farmers' current approach and is reported now in *Chapter 6*.

A practical reference test to detect bacteria involved in an IMI lacks. Thus, the true performance of new tests for mastitis in the laboratory as well as in the field cannot

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be assessed in terms of sensitivity (Se) and specificity (Sp), as misclassification of the reference test will affect the determined estimates. Therefore, latent class analysis was used to determine the test characteristics of the two tests used by farmers on-farm, and of the same two tests used in the laboratory. Their Se and Sp was determined for three diagnostic categories of mastitis pathogens without considering a test as the reference (*Chapter 7*).

A general discussion in *Chapter 8* summarizes and discusses the results as described in this thesis.

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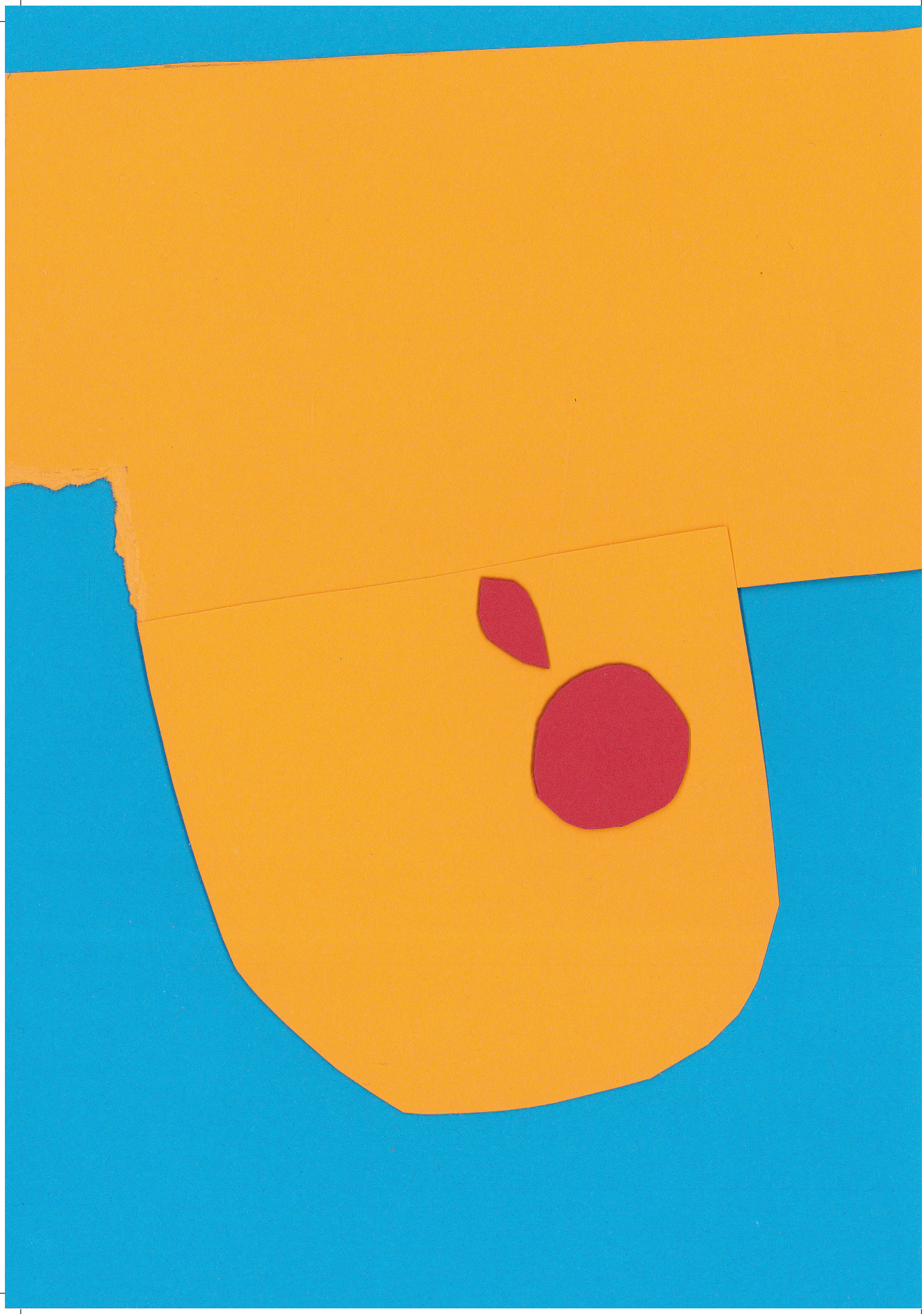

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Dutch dairy farmers' need for microbiological mastitis diagnostics

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Abstract

Although several microbiological mastitis diagnostic tools are currently available, dairy farmers rarely use them to base treatment decisions on. In this study, we conducted a telephone interview among 195 randomly selected Dutch dairy farmers to determine their current use of and their need for microbiological diagnostics for clinical mastitis (CM), subclinical mastitis (SCM), and dry-cow treatment (DCT), followed by the test characteristics they consider important. A structured questionnaire was used, based on face-to-face interviews previously held with other farmers. The answers were registered in a database and analyzed using descriptive statistics and univariable and multivariable models. Antimicrobial treatment decisions for CM, SCM, and DCT were mainly based on clinical signs and somatic cell count. In case of CM, 34% of farmers indicated that they currently submit milk samples for bacteriological culture (BC). This would increase to 71% if an on-farm test resulting in treatment advice within 12 h were available. For SCM, use would increase from 22 to 55%, and for DCT, from 7 to 34%, if the same 12-h test were available. For CM and DCT, the preferred test outcome was advice on which antibiotic to use, according to 58 and 15% of the farmers, respectively. For SCM, the preferred test outcome was the causative bacterium for 38% of the farmers. Farmers who currently submit CM milk samples for BC were 13.1 times more likely to indicate, as the preferred test outcome, advice on which antibiotic to use, compared with farmers who do not currently submit CM milk samples for BC. Fourteen percent of the farmers indicated not being interested at all in microbiological mastitis diagnostics for CM. For SCM and DCT, 27 and 55%, respectively, were not interested in microbiological mastitis diagnostics. Regarding test characteristics that farmers considered important, reliability was most often indicated (44–51% of the farmers). Additionally, a preferred time-to-result of ≤ 8 h for CM and ≤ 20 to 24 h for SCM and DCT and ≤ 7% false test outcomes were indicated as desired characteristics of microbiological mastitis diagnostics. Overall, a need seems to exist for microbiological mastitis diagnostic tests among Dutch dairy farmers, specifically for CM, and resulting in a treatment advice. The availability of a reliable diagnostic test, with a suitable time-to-result, will likely increase the use of microbiological mastitis diagnostics and eventually optimize antibiotic usage.

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Introduction

The main indications for using antimicrobial agents on dairy farms are the treatment and prevention of clinical mastitis (CM) and subclinical mastitis (SCM; Pol and Ruegg, 2007). Because the use of antimicrobial agents may lead to antimicrobial resistance (Levy and Marshall, 2004), limiting antibiotic usage based on microbiological diagnosis is advisable (Roberson, 2003). Additionally, the benefit of applying antimicrobial agents is debatable in some situations. For example, the cure rates of mild gram-negative coliform CM did not differ between groups of dairy cows that were treated with or without antimicrobial agents (Guterbock et al., 1993; Suojala et al., 2010). The same is true for SCM where the benefit of antibiotic treatment depends on the severity and duration of the infection (Barlow et al., 2009; van den Borne et al., 2010). Additionally, the preventive use of antimicrobial agents in dry-cow treatment (DCT) is under discussion in some countries (Scherpenzeel et al., 2014). Hence, for both treatment and prevention of IMI, a decision has to be made whether or not to use antimicrobial agents. Dependent on the legislation in a country, the decision to use antimicrobial agents is made by the veterinarian or the farmer. For prudent use of antimicrobial agents related to mastitis, determining whether susceptible bacteria are present through microbiological diagnosis of milk samples is critical (Lago et al., 2011a). The discussion on antibiotic usage, as well as the changing legislation, social pressure, and economic incentives of limited antibiotic usage, are factors likely to increase the role of microbiological mastitis diagnostics in the coming years.

Although several laboratory tools for microbiological diagnosis of IMI for dairy farmers and their veterinarians are available currently, these are rarely used to support treatment decisions (Owens et al., 1997; Lago et al., 2011a). The small number of milk samples submitted to bacteriological laboratories can be explained by the related costs, by the required effort of the farmer involved (Royster et al., 2014), and by the time-to-result (Neeser et al., 2006; Lago et al., 2011a). The current laboratory microbiological diagnostic methods are not considered suitable to base targeted treatment of CM in practice on, because of a time lag of > 24 h between sampling and result (Viora et al., 2014). Consequently, mastitis treatment decisions are usually made empirically or based on historic bacteriological culture and susceptibility results (Owens et al., 1997). To overcome the delay due to the long time-to-result, the use of on-farm mastitis diagnostics has expanded in countries such as the United States and Canada (Roberson, 2003; Cameron et al., 2013). With on-farm mastitis diagnostics, different categories of

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mastitis pathogens may be identified (Viora et al., 2014), leading to faster treatment decisions (Lago et al., 2011a,b; Royster et al., 2014) and selective use of antimicrobial agents in CM (Pinzón-Sánchez et al., 2011). In many countries in Europe, however, it is still common practice to treat all cases of CM with antimicrobial agents (Viora et al., 2014), which may be due to the lack of microbiological mastitis diagnostic tests considered suitable by farmers for making treatment decisions. To our knowledge, the needs of dairy farmers with respect to this type of tests have never been described. The aim of this study was to determine the Dutch dairy farmers' current use of, and their need for, microbiological mastitis diagnostics of CM, SCM, and DCT and to determine which test characteristics they consider important.

Materials and methods

Study design

A telephone interview was conducted among randomly selected Dutch dairy farmers using a structured questionnaire. The questionnaire was based on face-to-face interviews that were previously held with other farmers and are briefly discussed below. Based on that experience, the questions for the telephone interview were chosen from those used in the face-to-face interviews. These questions focused on subjects that came up as potentially important from the face-to-face interviews. The results of the telephone interviews were analyzed and are discussed in this paper.

Semi-structured face-to-face interviews

The individual face-to-face interviews were held by the first author with nonrandomly selected Dutch dairy farmers between October and December 2014, using a qualitative semi-structured questionnaire with open-ended questions. The first author is a veterinarian, which was not known by the farmers at the time of interview. The questionnaire was previously discussed with a communications expert and two mastitis experts. The goal of the face-to-face interviews was to gather a broad range of attitudes regarding mastitis and mastitis diagnostics, forming the base of the subsequent telephone questionnaire. The participants were selected with the goal of including farms with differences in characteristics such as herd size, milking system, farmers' focus on udder health, management style, and mastitis incidence. After interviewing 20 farmers, no new information was obtained and the interviews were stopped.

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Structured telephone interviews

Selection of farmers

In December 2014 and January 2015, 660 dairy farmers were randomly selected from a list of all 17,563 Dutch dairy farmers. The goal was to gather 200 participants. The farmers received a letter by mail with a short description of the study and the announcement that they might be approached by telephone for participation in a 30-min questionnaire on mastitis and microbiological mastitis diagnostics. The farmers were asked to look up their most recent bulk milk SCC, the number of CM cases in 2014, antibiotic usage in 2014 (animal daily dose, based on the national monitoring system; Speksnijder et al., 2015), and the prevalence of high-SCC cows (heifers ≥ 150,000 cells/mL, older cows ≥ 250,000 cells/mL; de Haas et al., 2008) at the last milk recording. Within 2 wk after the letters were sent, farmers were approached by telephone to ask whether they were willing to participate. If positive, either the interview was held directly or an appointment was made. If negative, the reason for being unwilling to participate was asked as well as two additional questions on the current herd size and perceived mastitis problems at their farm. Farmers with a herd size < 20 cows (aged > 2 yr) and farmers who intended to quit farming within the next 5 yr were excluded from participation.

Interview design

The questionnaire included 59 questions, divided over four sections, and took 20 to 45 min to complete. The interviews were held by the first author and two students with a background in farm animal husbandry; it was pretested on two farmers. Those two interviews were included in the analysis because no changes were made to the questionnaire after pretesting. The general part of the questionnaire included background information on the farmer and his farm, udder health consultancy services on the farm, and udder health characteristics. The specific part of the questionnaire was divided into a part on CM, a part on SCM, and a part on DCT. Each part held three sections with the same structure and discussed the farmers' current treatment and diagnostic approach, their needs for microbiological mastitis diagnostics, and the desired test characteristics of microbiological mastitis diagnostics. The questionnaire is available upon request from the corresponding author. Questions on the current treatment and diagnostic approach were open-ended to clarify the definitions used for determining cases and treatments for CM, SCM, and DCT.

To determine the need for microbiological mastitis diagnostics, both preferred and potential test outcomes and the intended use of microbiological mastitis diagnostics

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were discussed. As related to preferred test outcomes, farmers were asked which outcomes they expect to receive from such a test (open-ended question; more than one outcome could be mentioned). Additionally, three potential test outcomes were presented to the farmers to determine their need for each outcome: the first outcome was whether to treat with antimicrobial agents, the second was which antibiotic to use, and the third was whether to extend treatment (the latter test outcome was not discussed for DCT). The intended use of microbiological mastitis diagnostics was determined with the help of two hypothetical tests. Both hypothetical tests resulted in treatment advice and had a time-to-result of < 12 h. One of these tests was executed at the veterinary practice and the other one on the farm. To determine the desired test characteristics, the farmers were asked to indicate which characteristics they considered important for tests executed at the veterinary practice and on-farm (open-ended question; more than one characteristic could be mentioned). Subsequently, the farmers ranked four characteristics: speed, reliability, usability, and price (out-of-pocket cost), with 1 being the most important and 4 being the least important. For each of these characteristics, the overall mean was calculated to enable a comparison among them. Additionally, the farmers were asked what values they considered acceptable for those four characteristics; for example, what price were they willing to pay for a test.

Besides the open-ended questions, the standardized questionnaire contained closed questions, which consisted of scores on a Likert scale (Likert, 1932), and yes/no responses. The farmers were asked permission to use information on their herd size based on the Identification and Registration (I&R) census data (RVO, The Hague, the Netherlands) to be able to compare them with the average Dutch dairy farm. Results of the interview were entered in an online survey program (NetQ; http://www.netq-enquete.nl/nl/eng) and combined with the I&R data.

Data analysis

The representativeness of the participants was evaluated by comparing the means descriptors of the participating farms with the average Dutch dairy farm (Statistics Netherlands, The Hague/Heerlen, the Netherlands; de Koeijer et al., 2014) using a one-sample *t*-test. Test characteristics indicated by the farmers were evaluated using a paired *t*-test. Differences were considered statistically significant if $P < 0.05$.

To determine differences between farmers with respect to their opinion on microbiological mastitis diagnostics, regression models were used. Multivariable

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logistic regression models were used for CM, whereas univariable analysis was executed for SCM and DCT. The dependent variables consisted of the farmers' need for microbiological mastitis diagnostics and the desired test characteristics. The predictor variables evaluated for these two dependent variables were whether the udder health situation was discussed with the veterinarian; whether bacteriological culturing was executed; perceived mastitis problems at their farm; antibiotic usage based on animal daily dose; the percentage of high-SCC cows; bulk milk SCC; access to pasture; milking with an automatic milking system; age of the farmer; the average growth in herd size during the last 2 yr; the average herd size during the last 2 yr; and the average herd replacement rate during the last 2 yr.

Multivariable model selection for CM was conducted by including all variables in the model with a *P*-value < 0.25 (univariable). When two variables were highly correlated (correlation > 0.5) only the variable with the lowest *P*-value was included in the model. A backward selection procedure was followed, where the variables with the highest *P*-value in the model were deleted one by one, until only significant variables ($P < 0.05$) and confounders (difference in coefficients of ≥ 20%) remained in the model. Significant variables were identified by comparing the goodness of fit (log-likelihood) using the likelihood ratio test ($P < 0.05$). Interaction terms were added to the model and tested for statistical significance to investigate possible effect modification. During the analysis, a minimum of five per cell was demanded.

Results

Descriptive statistics

In total, 459 farmers were approached by telephone, of which 210 agreed to participate in the interview (46%). Of these 210 farmers, four farmers had fewer than 20 adult cows (average over the last 2 yr) and 11 farmers intended to quit farming within 5 yr and were therefore excluded. A total of 195 interviews were included in the analysis. Two hundred forty-nine farmers were not willing to participate for miscellaneous reasons, of which the time constraint was mentioned most frequently (37%). Of the farmers not willing to participate, 51% were willing to answer two questions: their mean current herd size was 90.3 adult cows (age > 2 yr) and 31% of them perceived mastitis problems on their farm.

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Table 1. Mean descriptors of the participating farms (no. of respondents in parenthesis) and the average Dutch dairy farm

Outcome	Participating farms		Average Dutch dairy farm
No. of adult cows (> 2 yr)	94.5[1]	(166)	85[2]
Organic farming (%)	2.1[3]	(192)	2[2]
Pasturing (%)	75[3]	(192)	76[2]
Full time labor units (no.)	1.6[3]	(192)	1.5[2]
Automatic milking system (%)	20.8[3]	(192)	18.7[4]
Farmer age (yr)	48[3*]	(191)	52[5]
Bulk milk somatic cell count (x 1,000 cells/mL)	157[3*]	(191)	217[6]
Cows with high somatic cell count (%)	11.6[3*]	(180)	18.9[6]
Animal daily dose	2.6[3]	(182)	2.4[2]
Average herd replacement rate per year (%)	24.0[1,7*]	(163)	25[5]
Average herd growth in no. of adult cows (> 2yr) per year (%)	3.7[1,7*]	(163)	2.3[5]

[1] Based on Identification and Registration (I&R) census data (RVO, The Hague, the Netherlands)
[2] Statistics Netherlands, The Hague/Heerlen, 2014
[3] Self-reported by the farmers
[4] Stichting KOM (2015)
[5] de Koeijer et al. (2014)
[6] GD Animal Health (2015)
[7] Difference in no. of cows > 2yr between the fourth trimester of 2012 and the third trimester of 2014
[*] $P < 0.05$

The bulk milk SCC and the percentage of cows with high SCC were lower for the 195 participating farmers than for the average Dutch dairy farm (Table 1). Over the last 2 yr, the mean herd size of the participants increased more and the herd replacement rate was lower than that of the average Dutch dairy farm. The participating farmers were slightly younger than the average Dutch dairy farmer.

The participating farmers reported an average of 15.7 cases of CM per 100 cows per year on their farms. Thirty-one percent of them perceived mastitis problems on their farm, which was the same as indicated by the farmers who were not willing to participate. The udder health situation is discussed with the veterinarian by 87% of the participating farmers, generally during regular herd visits (67% of farmers) or in case of mastitis-related problems (29% of farmers). None of the variables had a correlation > 0.5.

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Farmers' current treatment and diagnostic approach

Most farmers indicated that they did not treat every case of CM with antimicrobial agents, and they reported a variety of ways to determine whether to start such treatment. The most frequently reported indication to start an antibiotic treatment of CM cases was the presence of local signs such as clots or flakes in the milk or changes in the udder (31% of farmers). Nineteen percent of the farmers indicated that they started antibiotic treatment only when a cow with CM showed general signs such as illness; 28% used other criteria, such as the conductivity of the milk or failure of an alternative treatment. Bacteriological culture results were used by 2% of the farmers. Fifteen percent of the farmers indicated that they treated every cow with CM and 5% never treated a cow with CM with antimicrobial agents. The duration of the antibiotic treatment was generally based on label instructions (37% of farmers) or on the treatment protocol from the veterinarian (25% of farmers). Other farmers indicated that they took into account the recovery of cow when deciding on the duration of the treatment.

Subclinical mastitis is defined by most farmers as cows with a high individual SCC. Cut-off values used were, on average, 176,000 cells/mL for heifers [10th percentile (P10) 80,000; 90th percentile (P90) 275,000] and 284,000 cells/mL for older cows (P10: 150,000; P90: 400,000). Fifty-seven percent of the farmers never treated SCM during lactation. The other farmers indicated that they treated SCM during lactation always to sporadically. The most frequently used criteria for antibiotic treatment were a high SCC two to four times in a row (46% of farmers), a positive California Mastitis Test (22%), and a positive outcome of bacteriological culture (20%). The duration of an antibiotic treatment for SCM was based on the label instructions by 36% of the farmers or on the treatment protocol from the veterinarian (30% of the farmers).

Most farmers were satisfied with their current diagnostic approach of DCT (93%), where 85% of the farmers indicated that they did not use bacteriological diagnostics for DCT.

Thirty-four percent of the farmers indicated that they currently submitted milk samples of CM cases for bacteriological diagnosis always to regularly. For SCM and DCT, this was 22 and 7%, respectively. We detected a tendency for larger farms to submit milk samples of CM cases more frequently (data not shown).

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Need for mastitis diagnostics

Preferred test outcomes

For CM, test outcomes of interest were advice on which antibiotic to use, indicated by 58% of the farmers, and the causative bacterium, indicated by 53% of the farmers. Fourteen percent of the farmers indicated that they were not interested at all in microbiological diagnostics related to CM. For SCM, test outcomes of interest were the causative bacterium, indicated by 38% of the farmers, and advice on which antibiotic to use, indicated by 35% of the farmers. Twenty-seven percent of the farmers indicated that they were not interested at all in microbiological diagnostics related to SCM. For DCT, test outcomes of interest were advice on which antibiotic to use, indicated by 15% of the farmers, and the bacterium present, indicated by 11% of the farmers. Fifty-five percent of the farmers indicated that they were not interested at all in microbiological diagnostics related to DCT.

Potential test outcomes

Based on the presented potential test outcomes, 64% of the farmers expressed their need for a microbiological diagnostic test for CM in which the test outcome was advice on which antibiotic to use (Table 2). The need for a test resulting in advice on whether to treat with antimicrobial agents was expressed by 57% of the farmers. The lowest need was expressed for a test resulting in whether to extend an antibiotic treatment (38% of farmers). For SCM, 57% of farmers expressed their need for a test where the test outcome was advice on which antibiotic to use, and 31% expressed their need for a test resulting in whether to extend an antibiotic treatment. Farmers expressed the lowest need for microbiological mastitis diagnostics for DCT: 31% expressed their need for a test resulting in advice on which antibiotic to use. The need for a test resulting in whether antibiotic treatment is necessary for low-SCC cows at drying off was expressed by 13% of the farmers.

Of the farmers who expressed their need for a microbiological diagnostic test for CM resulting in advice on which antibiotic to use, 72% also expressed their need for such a test for SCM, and 38% for DCT. Of the farmers who expressed their need for a microbiological diagnostic test for CM resulting in whether antibiotic treatment is necessary, 62% also expressed their need for such a test for SCM, and 34% for DCT. Of the farmers who expressed their need for a microbiological diagnostic test for CM resulting in whether to extend treatment, 57% also expressed their need for such a test for SCM. Of the farmers who expressed no need for a microbiological diagnostic

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test for CM, 60% expressed no need for such a test for SCM, whereas 83% expressed no need for such as test for DCT.

Table 2. Farmers' needs for microbiological mastitis diagnostics (%) for the presented test outcomes, related to clinical mastitis (CM; *n* = 195), subclinical mastitis (SCM; *n* = 194) and dry-cow treatment (DCT; *n* = 193)

	Do you need a test result that determines whether an antibiotic treatment is necessary in case of CM or SCM during lactation and in case of low or high somatic cell count (SCC) for DCT?				**In case of an intended antibiotic treatment, do you need a test result that advise on the antibiotic to use in case of CM, SCM, or DCT?**			**Do you need a test result that determines whether to extend the treatment in case of CM or SCM during lactation?**	
			DCT						
Extent of interest	**CM**	**SCM**	**high SCC**	**low SCC**	**CM**	**SCM**	**DCT**	**CM**	**SCM**
Always	6.2	7.7	3.6	2.1	13.8	13.4	3.6	6.2	7.2
Often	23.6	21.1	11.4	4.7	29.2	25.3	14.5	15.9	10.8
Sometimes	27.2	23.2	11.9	5.7	20.5	18.6	13.0	16.4	13.4
Sporadic	12.3	9.3	6.2	4.7	10.8	5.2	6.2	8.7	5.7
Never	25.1	35.1	62.2	78.8	23.6	34.0	56.5	47.2	57.7
I don't know	5.6	3.6	4.7	4.2	2.1	3.6	6.2	5.6	5.2

Table 3. Farmers' expressed intended use (%) of a defined hypothetical test[1] executed at the veterinary practice or on-farm in case of clinical mastitis (CM; *n* = 195), subclinical mastitis (SCM; *n* = 194), or dry-cow treatment (DCT; *n* = 193)

	Veterinary practice			**On-farm**		
Extent of use	**CM**	**SCM**	**DCT**	**CM**	**SCM**	**DCT**
Always	7.7	3.1	1.6	13.9	11.3	4.2
Often	16.9	16.5	5.2	36.4	25.8	14.0
Sometimes	28.7	26.3	13.0	21.0	17.5	16.1
Sporadic	20.0	15.0	13.0	10.8	6.7	9.8
Never	21.5	30.9	56.5	15.9	33.0	43.5
I don't know	5.1	8.3	10.9	2.1	5.7	12.4

[1] Microbiological diagnostic mastitis test with a time-to-result of 12 h, and a treatment advice as the outcome

Intended use of mastitis diagnostics

Of the farmers, 53% expressed that they were willing to use (sometimes or always) the described hypothetical microbiological diagnostic test for CM if it were executed at

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the veterinary practice (Table 3). If executed on-farm, the intention was higher: 71% of the farmer expressed they were willing to use such a test (sometimes or always). For SCM and DCT, 46 and 20%, respectively, expressed that they were willing to use such a test at the veterinary practice, and 55 and 34%, respectively, expressed that they were willing to use an on-farm test.

Test characteristics

Reliability was indicated most often as an important characteristic for a microbiological diagnostic test of SCM and DCT (Table 4). For CM tests executed at the veterinary practice, however, time-to-result was indicated most often as an important test characteristic. When ranking the importance of test characteristics, reliability was considered most important for CM, SCM, and DCT. For CM, time-to-result was of second importance, followed by price (out-of-pocket cost) and usability, which were considered equally important (Table 5). For SCM, time-to-result, price (out-of-pocket cost), and usability were considered equally important. For DCT, time-to-result was considered significantly less important than price (out-of-pocket cost) and usability.

Table 4. Percentage of farmers who indicated the mentioned test characteristics of microbiological mastitis diagnostics as important for clinical mastitis (CM; *n* = 195), subclinical mastitis (SCM; *n* = 194), and for dry-cow treatment (DCT; *n* = 193)

	Test characteristics		
Test location	**Reliability**	**Time-to-result**	**Price**[1]
Veterinary clinic			
CM	46	48	23
SCM	47	30	21
DCT	44	13	17
On-farm			
CM	48	41	14
SCM	51	27	24
DCT	48	15	21

[1] Price = Out-of-pocket costs

A test was considered reliable if the percentage of false test outcomes was < 7% for CM, SCM, and DCT. A hands-on time of 7.5 to 10 min was considered suitable for executing a microbiological diagnostic test for CM, SCM, and DCT (P10: 3 min; P90: 15 min). Farmers considered a median of 8 h (P10: 2 h; P90: 12 h) an acceptable time-to-result for CM. For SCM, a median of 20 h was considered an acceptable time-to-result (P10: 4 h; P90: 24 h)

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and for DCT, a median of 24 h (P10: 5 h; P90: 48 h). Farmers considered out-of-pocket costs of €15 for CM (P10: €5; P90: €50) and SCM (P10: €5; P90: €30) acceptable. For DCT, median out-of-pocket costs of €10 (P10: €3; P90: €25) was considered acceptable.

Clinical mastitis: farmer characteristics and need for microbiological mastitis diagnostics

Not surprisingly, the greatest need for microbiological mastitis diagnostics resulting in advice on which antibiotic to use was indicated by farmers who were already submitting milk samples in case of CM [odds ratio (OR) 13.1]. Farmers who perceived mastitis as a problem on their farm indicated a greater need for microbiological mastitis diagnostics resulting in whether it is advisable to start an antibiotic treatment (OR 2.7) or to extend an antibiotic treatment (OR 2.9). Apart from that, farmers with cows that had access to pasture indicated a greater need for a test resulting in advice on which antibiotic to use in case of an intended antibiotic treatment of CM (OR 3.1; Table 6). The proportion of variance in the need for microbiological mastitis diagnostics explained by the model was 24% for a test resulting in advice on which antibiotic to use.

With respect to the desired test characteristics, farmers who perceived mastitis as a problem on their farm were more likely to consider reliability of an outcome more important than other farmers, for both a test performed at the veterinary practice (OR 2.6; 95% confidence interval (CI): 1.30–5.36) and an on-farm test (OR 2.4; 95% CI: 1.27–4.48). As shown in Table 7, farmers with increased herd size during the last 2 yr were likely to consider time-to-result important more often than other farmers, for both a test at the veterinary practice (OR 1.15) and an on-farm test (OR 1.10).

Table 5. Ranking[1] of test characteristics by dairy farmers, expressed as means (SD) of scores per characteristic for tests for clinical mastitis (CM; *n* = 195), subclinical mastitis (SCM; *n* = 194) and dry-cow treatment (DCT; *n* = 193)

	Test characteristic			
Item	**Reliability**	**Time-to-result**	**Price[2]**	**Usability**
CM	1.5 (0.77)^{a,C}	2.2 (0.87)^{c,B}	3.3 (0.87)^{a,A}	3.1 (0.92)^{a,A}
SCM	1.3 (0.64)^{b,B}	2.9 (0.93)^{b,A}	2.9 (1.03)^{b,A}	2.8 (0.85)^{b,A}
DCT	1.3 (0.70)^{C0}	3.3 (0.86)^{a,A}	2.7 (1.04)^{c,B}	2.7 (0.81)^{b,B}

$^{a-c}$Values with different superscripts within columns differ significantly ($P < 0.05$)
$^{A-C}$Values with different superscripts within rows differ significantly ($P < 0.05$)
[1] Mean ranks: 1 is most important, 4 is least important
[2] Price = Out-of-pocket costs

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Discussion

This is the first study to determine the needs of dairy farmers for mastitis diagnostics. We found that a need for mastitis diagnostics was present among Dutch dairy farmers, with a preference for tests that are available on-farm and have a short time-to-result. The use of microbiological mastitis diagnostics would be twice as high as the general use of mastitis diagnostics if current tests fulfilled these criteria. It is important to note that a selection bias toward farmers interested in udder health is present in this study (Pennings et al., 2002), as indicated by the lower-than-average bulk milk SCC and prevalence of high-SCC cows. Thus, the quantitative results of this study may slightly overestimate the needs of dairy farmers. The younger age of the participating farmers and the larger herd size of the farms were as expected, because younger farmers and farmers with larger herd sizes are more often willing to respond to questionnaires (Pennings et al., 2002). Because perceiving mastitis problems is an important cue to action (Jansen et al., 2009) and, given the fact that the farmers who were not willing to participate perceived mastitis problems on their farms to the same extent as participating farmers, we consider the qualitative results of this study to be representative.

Although there are limitations on conducting a telephone interview (nonresponse bias, lack of body language), it gave the opportunity to approach a large number of people and to quantify the responses of the face-to-face interviews without losing the opportunity to elucidate ambiguities, making it possible to determine the needs of the farmers.

In this study, farmers expressed their need for rapid microbiological mastitis diagnostics. To date, bacteriological culturing has a time-to-result of ≥ 24 h. Testing by PCR is quicker but is not executed on farm and has the disadvantage of requiring sample transport to a laboratory. In our study, only 2% of the farmers indicated that they used microbiological culture results as the basis for treatment decisions. Although waiting 24 h for culture results has no negative effect on cure rates or cow survival (Lago et al., 2011a,b), farmers find it difficult to postpone treatment decisions (Neeser et al., 2006). A time-to-result of 12 h is considered an improvement compared with the current diagnostics available because the intended use of the hypothetical tests was twice as high as the current use of mastitis diagnostics in general. The need for fast mastitis diagnostics was found to be most explicit for CM, where farmers considered a time-to-result of ≤ 8 h acceptable,

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Table 6. Dairy farmers' need for microbiological mastitis diagnostics for clinical mastitis subdivided by farm characteristics using three potential test outcomes

	Do you need a test result that determines whether an antibiotic treatment is necessary in case of clinical mastitis? (*explained variability = 7%*)			**Do you need a test result that advises which antibiotic to use in case of an intended antibiotic treatment in case of clinical mastitis?** (*explained variability = 24%*)			**Do you need a test result that determines whether to extend treatment in case of clinical mastitis?** (*explained variability = 11%*)		
Item	*n*	**OR**[1]	**95% CI**	*n*	**OR**	**95% CI**	*n*	**OR**	**95% CI**
Acces to pasture									
No				21	Referent				
Yes				81	3.09*	1.23-7.80			
Current use of bacteriological culturing					***				
Always/often				18	13.08***	3.08-55.60	10	1.59	0.48-5.32
Sometimes				23	8.89***	2.63-30.04	14	2.11	0.72-6.15
Sporadic				42	7.07***	2.59-19.34	26	2.45	0.96-6.23
Never				19	Referent		12[2]	Referent	
Average growth in herd size during last 2 yr				100	1.11*	1.03-1.20			
Perceiving mastitis problems									
No	45	Referent		63[2]	Referent		33	Referent	
Yes	26	2.72*	1.12-6.60	39	2.11	0.79-5.63	29	2.91**	1.30-6.52
ADD[3]	68	1.41*	1.06-1.88				61[2]	1.22	0.93-1.59
Bulk milk somatic cell count				102	1.01**	1.00-1.02			

[1] OR = odds ratio
[2] Confounder
[3] ADD = animal daily dose, antibiotic usage based on national monitoring program
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

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whereas a longer time-to-result (≤ 20 and ≤ 24 h, respectively) for SCM and DCT was considered acceptable. Although the answers of the farmers might be influenced by the predefined tests, which had a time-to-result of 12 h, the indicated desired time-to-result differed from the predefined tests for the three indications. The current available on-farm microbiological tests could be of interest for the farmers, because these tests have a time-to-result of 18 to 24 h. These on-farm tests, however, are rarely used in the Netherlands. This may be because farmers are not aware of those tests, that herd sizes are too small for the farmers to collect enough milk samples and gain experience with the tests, or that they need the encouragement of other farmers or veterinarians who are enthusiastic about such diagnostic tests. The importance of social pressure was found in an earlier study on preventive measures regarding mastitis management (Jansen et al., 2009). Furthermore, a time-to-result < 24 h may not be necessary for SCM and DCT diagnostics. Although we could assume that farmers are aware of this, they indicated that they appreciated a shorter time-to-result. This is likely based on emotions rather than on rational considerations because these cows have been infected for some time. Earlier studies showed that good stockman ship is important to farmers, which is not always based on rational considerations (Jansen et al., 2009; Swinkels et al., 2015).

Regarding CM and DCT, farmers were most interested in advice on which antibiotic to use rather than identification of the causative agent as a test outcome. In addition to farmers who already submit milk samples for bacteriological culture, this need is specifically indicated by farmers who pasture their cows. This may be because farmers pasturing their cows have a different attitude toward management than other farmers. With respect to SCM, however, farmers were most interested in the causative bacterium. This may be due to use of diagnostic results in management decisions at the herd level, such as focusing on contagious or environmental mastitis pathogens (De Vliegher et al., 2012) or at the cow level, such as culling or segregation. A continuous strategy of early detection of SCC with microbiological diagnosis directly followed by treatment based on the obtained results could improve cure rates of SCM (Barkema et al., 2006). Currently, however, this type of diagnostic approach is not used much: only 22% of the farmers indicated that they submitted milk samples for SCM. If farmers were willing to increase the use of microbiological mastitis diagnostics, as indicated in this study, this continuous strategy could result in a lower incidence of high-SCC animals in the herd and eventually in lower CM incidence. Furthermore, the use of on-farm mastitis diagnostics may result in informed treatment decisions and thus in limited usage of antimicrobial agents (McCarron et al., 2009).

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Table 7. Time-to-result as a desired test characteristic of microbiological mastitis diagnostics for clinical mastitis (CM) according to dairy farmers subdivided by different farm characteristics

	Veterinary clinic (explained variability = 13%)			On-farm (explained variability = 4%)		
	n	OR[1]	95% CI	*n*	OR	95% CI
Average growth in herd size over last 2 yr	76	1.15***	1.07-1.24	64	1.10**	1.03-1.18
Currently submitting milk samples for bacteriological culture in case of CM		*				
Always/often	11	2.36	0.87-6.87	-	-	-
Sometimes	21	4.57**	1.64-12.77	-	-	-
Sporadic	27	1.99	0.86-4.57	-	-	-
Never	18	Referent		-	-	-
Farmers' age[2]	76	0.96*	0.92-0.99	-	-	-

[1] OR = odds ratio
[2] Year of birth
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The percentage of farmers currently submitting CM and SCM milk samples to laboratories is comparable with that reported in earlier studies (Hoe and Ruegg, 2006). In the Nordic countries, however, CM milk samples for microbiological diagnosis are submitted more often, although some between-country variation exists (Espetvedt et al., 2013). Our finding that farmers with larger herds submitted milk samples more frequently is in line with previous findings (Hoe and Ruegg, 2006). Furthermore, farmers with increasing herd sizes seem to have a greater need for a test resulting in advice on which antibiotic to use. With increasing herd sizes worldwide (Barkema et al., 2015), the need for microbiological mastitis diagnostics may increase.

Farmers reporting higher antibiotic usage more often indicated their need for a CM test indicating whether an antibiotic treatment was necessary (Table 6). This may suggest that these farmers consider microbiological mastitis diagnostics a potential way to reduce their antibiotic usage. Furthermore, one-third of the farmers were interested in a test resulting in whether or not to extend treatment of CM. Currently, the length of the treatment is generally based on the treatment protocol from the veterinarian, the label instructions of the antibiotic used, or the recovery of the cow. Although cure rates of CM may increase by extending treatment (Pinzón-Sánchez et al., 2011), and many farmers extend treatment (Swinkels et al., 2015), it is not always advisable to do so (Swinkels et al., 2013). The fact that one-third of the farmers were interested in a

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test to decide on extending treatment seems to indicate they are aware of the urgency of prudent antibiotic usage.

Worldwide, there is increasing public attention on antibiotic usage, which may lead to expanded requirements for applying antibiotic treatments in animal husbandry. Because reducing antibiotic resistance is one of the challenges of today's dairy industry (Barkema et al., 2015), microbiological mastitis diagnostics may be a useful tool in mastitis treatment decisions and may lead to more prudent antibiotic usage (Pinzón-Sánchez et al., 2011). Farmers expressed their need for reliable mastitis diagnostics, preferably on-farm, with a short time-to-result, and with an advice on which antibiotic to use as the outcome.

Concluding, Dutch dairy farmers need microbiological mastitis diagnostics, and they expressed their willingness to use that type of test for CM, SCM, and DCT more frequently than they currently do. Specifically for CM, farmers currently submitting milk samples for bacteriological culture and farmers perceiving mastitis problems expressed their need for a test resulting in advice on the antibiotic to use. The farmers expressed their need for a reliable, affordable diagnostic microbiological mastitis test that is preferably executed on-farm, does not have many false results, and has a time-to-result ≤ 8 h.

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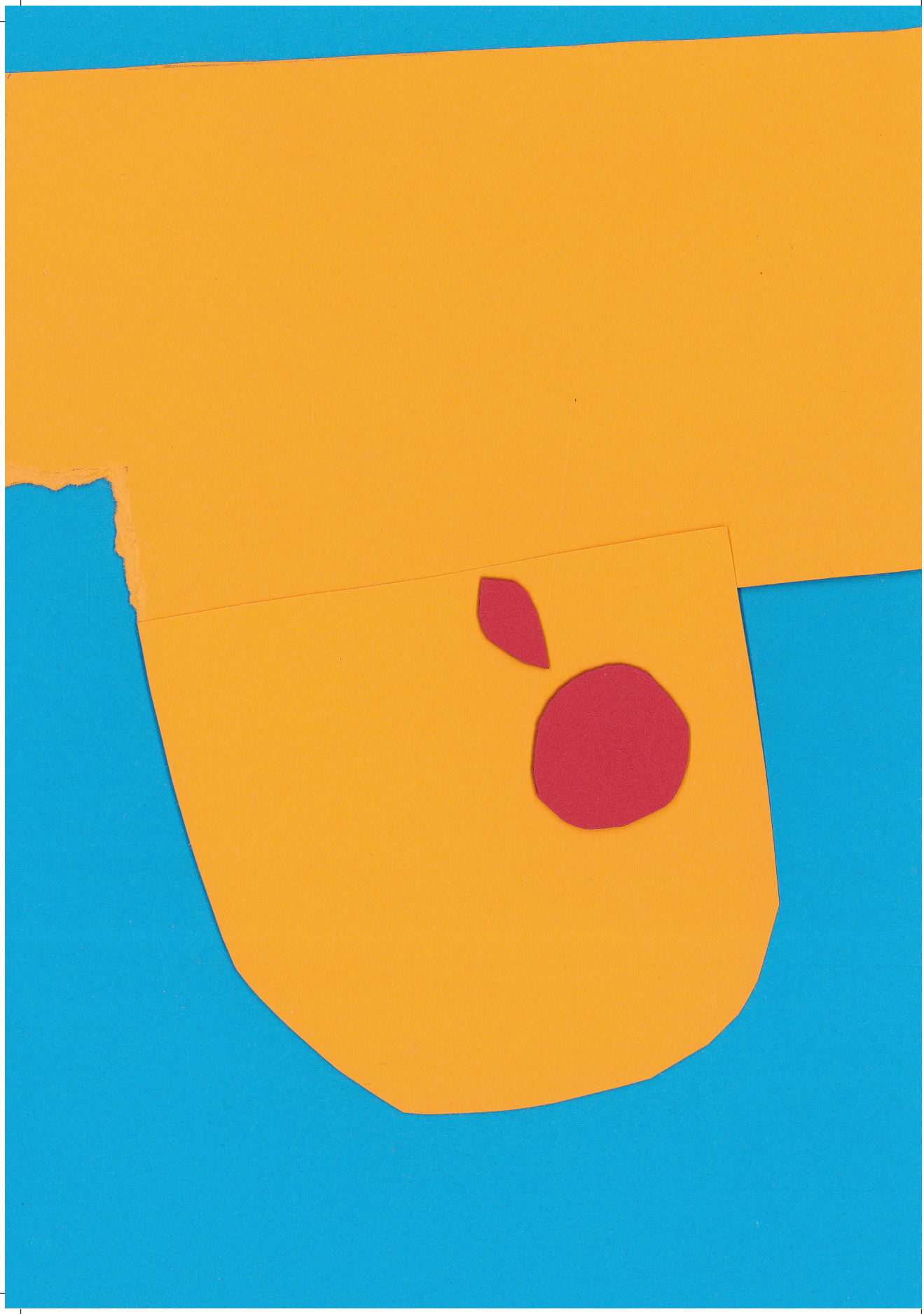

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Agreement between four commercial diagnostic tests and routine bacteriological culture of milk to determine the udder infection status of dairy cows

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Abstract

Mastitis is usually treated based on clinical signs or somatic cell count information rather than on results of bacteriological culture of milk. In many countries an optimal mastitis treatment is considered important from the perspective of therapy efficacy, prudent antimicrobial use and farm economics. Farmers can optimize their mastitis treatment decisions if they know whether and which mastitis pathogen is involved. Information on the mastitis pathogen involved can be acquired from diagnostic mastitis tests such as culture-based tests. This study aimed to determine the agreement of four commercial culture-based mastitis tests with routine bacteriological culture of milk to determine the intramammary infection status of a quarter or cow. The commercial culture-based tests evaluated in this study were CHROMagar Mastitis (CHROMagar, France), Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, USA), Minnesota Easy Culture System II Tri-plate (University of Minnesota, USA), and VétoRapid (Vetoquinol, the Netherlands). We used 866 prospectively collected milk samples, routinely submitted to the bacteriological laboratory of GD Animal Health for routine bacteriological culture of milk from April to June 2016. Samples were cultured on routine bacteriological culture of milk and on the commercial culture-based tests. We calculated the agreement beyond chance of each commercial culture-based test result with the result of routine bacteriological culture using 2x2 contingency tables. Furthermore, inter-reader agreement was determined for 597 samples read by two masked readers. The agreement of the four commercial culture-based mastitis tests with routine bacteriological culture of milk for Gram-positive bacteria ranged from 0.14 (95% CI 0.11-0.16) using Hardy Diagnostics Mastitis Triplate to 0.25 (95% CI 0.22-0.28) using Minnesota Easy Culture System II Tri-plate. The agreement for Gram-negative bacteria was approximately 0.70 (95% CI 0.66-0.74) for all four commercial culture-based tests. The agreement for no growth ranged from 0.22 (95% CI 0.19-0.25) using Hardy Diagnostics Mastitis Triplate to 0.34 (95% CI 0.31-0.38) using VétoRapid. This category was affected by prevalence and bias as the prevalence adjusted and bias adjusted kappa ranged from 0.63 (95% CI 0.56-0.69) using CHROMagar Mastitis to 0.68 (95% CI 0.62-0.74) using Hardy Diagnostic Mastitis Triplate. Agreement between readers was almost perfect. Although only for Gram-negative bacteria a good agreement was found between commercial culture-based tests and routine bacteriological culture of milk, and further on-farm evaluations are needed to determine the effect of these findings on udder health, commercial culture-based tests are of added value to support decisions whether and how to treat cows with mastitis.

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Introduction

Mastitis, an inflammation of the mammary gland mainly caused by bacteria, is the indication for which most antimicrobials are used on dairy farms (Pol and Ruegg, 2007). Currently, most cows with mastitis are treated with antimicrobials (Pol and Ruegg, 2007; Santman-Berends et al., 2015), even though some may not need antimicrobials (Roberson, 2003; Barkema et al., 2006; Pinzón-Sánchez et al., 2011). From a perspective of both prudent antimicrobial use as well as of limiting unnecessary costs, antimicrobials should preferably only be used in cases where an intramammary infection (IMI) can be confirmed, and, if possible, where probability of cure is high (Barkema et al., 2006; Krömker and Leimbach, 2017). Thus, for each mastitic cow one needs to decide whether treatment with antimicrobials is required (Royster and Wagner, 2015).

The general used parameters to decide on treatment like signs of mastitis, history of mastitis, or stage of lactation are not distinct enough to really target mastitis treatment (Pinzón-Sánchez et al., 2011). A targeted treatment can only be made if the causative pathogen is known, making diagnostic testing inevitable (Roberson, 2003). Additionally, farmers have indicated to be interested in additional testing to know the causative pathogen for udder health-related problems (Griffioen et al., 2016).

A broad range of diagnostic tests is available to determine the IMI status of a cow. However, tests like electronic conductivity measures or California Mastitis Test provide insufficient information to target treatment. Furthermore, laboratory tests are considered impractical due to the long time lag between sampling and test results and are therefore used infrequently (Griffioen et al., 2016).

Over the years commercial culture-based mastitis tests have been developed (Ganda et al., 2016; Leimbach and Krömker, 2018). Several laboratory studies evaluated the accuracy of commercial culture-based mastitis tests like Minnesota Easy Culture System II Bi- and Tri-plate, VétoRapid, and Petrifilm plates. These studies showed that commercial culture-based mastitis tests generally were able to categorize mastitis cases into treatment groups or to indicate mastitis-causing bacteria (McCarron et al., 2009; Cameron et al., 2013; Royster et al., 2014; Viora et al., 2014; Ferreira et al., 2018). Furthermore, they inform on the causative bacterium within 24 hours, and helped to decide on mastitis treatment without affecting cure rate, but with reducing the antimicrobial use (Leslie et al., 2005; Lago et al., 2011a). Nevertheless, a number of

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commercial culture-based mastitis tests are available with undetermined diagnostic accuracy.

Four commercial culture-based mastitis tests were selected to be evaluated in this study: CHROMagar Mastitis (CHROMagar), Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics), Minnesota Easy Culture System II Tri-plate (University of Minnesota), and VétoRapid (Vetoquinol). These four commercial culture-based tests were selected based on their commercial availability, their potential to be used on-farm, and the differences in utilized media. CHROMagar Mastitis and VétoRapid utilize chromogenic media substrates while Hardy Diagnostics Mastitis Triplate and Minnesota Easy Culture System II Tri-plate utilize more conventional culture media, including for example Edwards medium. The envisaged role for these tests is to diagnose an IMI on-farm. This information could help farmer or veterinarian to decide on udder health-related treatments in addition to already available information.

This study aimed to determine the agreement of four commercial culture-based mastitis tests with routine bacteriological culture of milk to determine the IMI status of a quarter or cow.

Materials and methods

Samples

Milk samples were prospectively collected from samples sent to the bacteriological laboratory of GD Animal Health (Deventer, the Netherlands) for routine bacteriological culture of milk between 4 April and 22 June 2016. Samples routinely sent in were considered eligible while samples sent in for research projects purpose were excluded. No clinical information was available on the cows the milk was collected from since such information is not requested at submission. Samples were cultured according to the routine procedure for bacteriological culture of milk, and by using each of the four commercial culture-based mastitis tests on the same day as far as possible. Otherwise samples were frozen at – 20 °C. When possible, somatic cell count (SCC) was determined.

Routine bacteriological culture of milk

Routine bacteriological culture of milk was performed at the bacteriological laboratory of GD Animal Health according to NMC guidelines (NMC, 1999). In brief, from each

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sample, 0.01 mL was inoculated onto 6% sheep blood agar (Biotrading, Mijdrecht, the Netherlands). Presumptive growth of mastitis-causing pathogens was examined after incubation for 18 to 24 h at 37 °C (under aerobic conditions) and again after 48 h. Identification of presumptive mastitis-causing pathogens was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper Microflex LT (Bruker Daltonics GmbH, Germany) (Barreiro et al., 2010). Milk samples that tested negative with standard culture procedure and having a SCC above 200,000 cells/mL were cultured again onto sheep blood agar following a combination of freezing and pre-incubation (Sol et al., 2002). An IMI was defined as a pure culture or predominance of one or two types of presumptive mastitis-causing pathogens with growth of at least six (in case of a pure culture) or more than ten (when more than one type was present) colonies on the plate. In case of growth of *Staphylococcus aureus*, *Streptococcus agalactiae*, or hemolytic streptococci the presence of already one colony was considered as an IMI. No growth was defined as no growth of presumptive mastitis-causing pathogens. Contamination was defined as growth of more than two phenotypically different colony types, without a dominant presumptive mastitis-causing pathogen. Then an IMI with that presumptive mastitis-causing pathogen was considered present and the sample was not considered contaminated. The SCC was determined using fluorescence flow cytometry (CombiScope 600, Delta Instruments, Drachten, the Netherlands) (ISO 13366-2|IDF 148-2:2006, 2006).

Commercial culture-based mastitis tests

All four commercial culture-based mastitis tests were inoculated with a new sterile cotton swab after mixing the milk sample gently and dipping the swab in the milk for eight to ten seconds to become fully saturated. The commercial culture-based mastitis tests were incubated and read after 18 h to 24 h, according to the manufacturer's guidelines (Minnesota Easy Culture System User's Guide, 2013; CHROMagar Mastitis version 2, 2014; Hardy Diagnostics Instructions for use Mastitis Triplate, 2016; VétoRapid Mastitits-Schnelltest, 2014). The commercial culture-based tests were regarded positive for IMI when one or more different colony morphologies grew. All different colony morphologies were identified visually as precise as possible, without further confirmation. When the colony could not visually be indicated to bacterial species level the reader reported the bacterial group or when that was not possible either 'Gram-positive' or 'Gram-negative' was noted. If more than two different colony morphologies were present on a test, 'contamination' was added in the report. When no bacterial

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growth was noticed 'no growth' was reported. Additionally, readers noted whether or not they were uncertain about the result.

CHROMagar Mastitis

CHROMagar Mastitis (CHROMagar, Paris, France) consists of two agars in two different Petri dishes. One agar specific for Gram-positive bacteria (with peptone and yeast extract, salt, and a chromogenic mix), and one specific for Gram-negative bacteria (with peptone and yeast extract, and a chromogenic mix). Both agars were incubated at 37 °C according to the manufacturer's guidelines (CHROMagar Mastitis version 2, 2014). Results of both plates were read independent of each other and were combined into a single result for each milk sample to analyze the accuracy of CHROMagar Mastitis.

Hardy Diagnostics Mastitis Triplate

Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA) consists of one Petri dish with three separate sections (triplate). One section is for total bacterial growth (a Tryptic Soy Agar with sheep blood), one is specific for streptococcal growth (a modified TKT agar with esculin), and one for Gram-negative growth (a modified MacConkey agar). The Hardy Diagnostics Mastitis Triplate was incubated at 35 °C according to the manufacturer's guidelines (Hardy Diagnostics Instructions for use Mastitis Triplate, 2016).

Minnesota Easy Culture System II Tri-plate

Minnesota Easy Culture System II Tri-plate (University of Minnesota, St. Paul, MN, USA) consists of one Petri dish with three separate sections (triplate). One section is specific for Gram-positive growth (factor agar), one for streptococcal growth (modified TKT agar), and one for Gram-negative growth (MacConkey agar). The Minnesota Easy Culture System II Tri-plate was incubated at 37 °C according to the manufacturer's guidelines (Minnesota Easy Culture System User's Guide, 2013).

VétoRapid

VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), consists of one Petri dish with three separate sections (triplate). One section is specific for staphylococci (modified mannitol salt agar), one for streptococci (modified Edwards agar containing crystal violet and polymyxin B), and one for Gram-negative bacteria (agar containing bile salts and vancomycin to prevent Gram-positive growth). VétoRapid was incubated at 37 °C according to the manufacturer's guidelines (VétoRapid Mastitis-Schnelltest, 2014).

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Readers of commercial culture-based tests

All commercial culture-based tests were read by one of five readers. A number of samples was read by a second reader to determine the level of agreement beyond chance between two readers. Readers were blinded to the results of routine bacteriological culture of milk and to each other's results. However, readers were not blinded to the results of the different commercial culture-based tests read by themselves. The commercial culture-based tests were read per test instead of per sample to diminish the influence of prior results of other commercial culture-based tests. Readers of routine bacteriological culture of milk were blind for commercial culture-based test results.

One of five readers was a laboratory technician having experience reading bacteriological culture results of milk samples, three readers lacked experience reading bacteriological culture results of milk samples: two of them were laboratory technicians and one was an intern, and one was a veterinarian having limited experience reading bacteriological culture results of milk samples. None had specific experience reading commercial culture-based tests.

Statistical analysis

Results of commercial culture-based mastitis tests were digitalized using NetQ (Collector 2015.Q2, Survalyzer, Utrecht, the Netherlands). Results of routine bacteriological milk culture as well as those of the four commercial culture-based mastitis tests were combined into one dataset. Only samples having a result of all four commercial culture-based tests and of routine bacteriological milk culture were used.

Results of all tests were categorized as shown in Table 1. For example, for routine bacteriological milk culture the category 'Gram-positive' comprised *Staphylococcus* spp. including *S. aureus* and coagulase negative staphylococci (CNS), *Streptococcus* spp. including *Streptococcus uberis*, *S. agalactiae* and *Streptococcus dysgalactiae*, and other Gram-positive bacteria, whereas 'Gram-negative' comprised lactose-fermenting coliforms including *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp. (KEC), non-lactose-fermenting Gram-negative bacteria including *Pseudomonas* spp., and other Gram-negative bacteria. For CHROMagar Mastitis the category 'Gram-positive' comprised *S. aureus*, *S. uberis*, *S. agalactiae* and other Gram-positive bacteria, the category 'Gram-negative' comprised *E. coli*, KEC, *Pseudomonas* spp. and other Gram-negative bacteria.

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Table 1. Routine bacteriological milk culture results of all milk samples used in study, of milk samples originating from quarters suspected of mastitis, and of milk samples with SCC ≤ 200,000 cells/mL

	All milk samples[1] (*n* = 866)		Mastitis samples[1,2] (*n* = 671)		SCC ≤ 200[1] (*n* = 141)	
	n	%	*n*	%	*n*	%
1 different colony morphology	571	65.9	459	68.7	82	58.2
2 different colony morphologies	49	5.7	35	5.2	7	5.0
≥ 3 different colony morphologies	106	12.2	73	10.9	24	17.0
No growth	140	16.2	104	15.6	28	19.9
Gram-positive bacteria	409	47.2	302	45.2	81	57.4
Staphylococcus spp.	209	24.1	151	22.6	46	32.6
S. aureus	128	14.8	97	14.5	26	18.4
CNS[3]	84	9.7	56	8.4	21	14.9
Streptococcus spp.	149	17.2	114	17.1	29	20.6
S. uberis	82	9.5	65	9.7	14	9.9
S. agalactiae	2	0.2	2	0.3	0	0.0
S. dysgalactiae	42	4.8	33	4.9	7	5.0
Other Gram-positive bacteria	73	8.4	54	8.1	11	7.8
Enterococcus spp.	16	1.8	10	1.5	2	1.4
Gram-negative bacteria	227	26.2	202	30.2	10	7.1
Lactose-fermenting bacteria	187	21.6	167	25.0	7	5.0
Escherichia coli	146	16.9	131	19.6	7	5.0
Klebsiella spp.	41	4.7	36	5.4	0	0.0
KEC[4]	70	8.1	60	9.0	3	2.1
Non-lactose-fermenting bacteria	11	1.3	11	1.6	0	0.0
Pseudomonas spp.	5	0.6	5	0.7	0	0.0
Other Gram-negative bacteria	6	0.7	6	0.9	0	0.0

[1] Results of samples with up to two different colony morphologies were included in the specification to bacterial species
[2] Mastitis samples comprised milk samples with abnormal milk appearance and those with SCC > 200,000 cells/mL
[3] CNS=coagulase negative staphylococci
[4] KEC=*Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp.

Three types of milk samples were examined: 1) all samples, 2) mastitis samples, a subset of one being milk samples with abnormal milk appearance or SCC > 200,000 cells/mL, and 3) low SCC samples, a subset of one being milk samples with SCC ≤ 200,000 cells/mL. Results of each commercial culture-based test were compared with results of routine

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bacteriological milk culture to calculate the agreement beyond change as expressed by kappa (Fleiss, 1971). When a milk sample was read by two readers within a commercial culture-based test, then one result was randomly selected. The random selection was repeated ten times for each commercial culture-based test. The obtained agreements were averaged per commercial culture-based test.

Agreement was computed for four diagnostic categories: no growth, Gram-positive bacteria, Gram-negative bacteria, and contamination as well as for each bacterial species or bacterial group that could be identified on the specific commercial culture-based mastitis test (Table 2). Additionally, kappa value was computed to determine the agreement between two commercial culture-based tests, and to determine the inter-reader agreement using the milk samples that were read by two readers. Results agreed when both tests or readers under comparison indicated the presence or absence of the diagnostic category or bacterial species or group. Results disagreed when one test or reader indicated a diagnostic category or bacterial species or group as present while the other test or reader indicated the diagnostic category or bacterial species or group as absent, or vice versa.

Kappa values were calculated with Stata 14.1 (StataCorp, 2015) and accounted for two or more readers since the identity of the readers differed (Fleiss, 1971). Confidence intervals were calculated following the formula $\kappa \pm 1.96$ S.E. with S.E.$=\sqrt{\kappa(1-\kappa)/n}$, with κ being kappa value and n the sample size. The prevalence adjusted and bias adjusted kappa (PABAK) was calculated using the formula $2P_o - 1$, with P_o being the proportion of observed agreement calculated as $P_o = {}^{a+d}/_{n}$ (Sim and Wright, 2005). With a being the number of positive agreed results and d the number of negative agreed results for the commercial culture-based test and routine bacteriological culture of milk. For PABAK confidence intervals were calculated following the formula $PABAK \pm 1.96 \sqrt{VAR}$ with $VAR = {}^{4P_o(1-P_o)}/_{n}$ (Byrt et al., 1993, Looney and Hagan, 2008). For both kappa and PABAK differences were considered significant when the 95% CI of two commercial culture-based tests were not overlapping within sample type per diagnostic category. For both kappa and PABAK the guideline according to Landis and Koch (1977) was used to determine the magnitude of agreement. Therefore, a value of > 0.80 was considered to be almost perfect, > 0.60-0.80 substantial, > 0.40-0.60 moderate, > 0.20-0.40 fair, > 0.00-0.20 slight, and ≤ 0.00 poor.

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We calculated the required sample size using the following formula (Watson and Petrie, 2010):

$$4\frac{(1-\kappa)}{W^2}\left((1-\kappa)(1-2\kappa)+\left(\frac{\kappa(2-\kappa)}{2\pi(1-\pi)}\right)\right)1.96^2$$

We assumed a prevalence (π) of 0.1 for the least prevalent diagnostic category no growth, accepted a 95% CI width (*W*) of 0.1, and wanted to estimate the sample size to give an almost perfect agreement between the commercial culture-based tests and routine bacteriological milk culture and therefore used an anticipated value for κ of 0.8. The required number of samples was 622.

Results

Between 4 April and 22 June 2016, 1472 milk samples were sent in for routine bacteriological milk culture of which 1447 were eligible to be included in the study (Figure 1). In total 866 milk samples were cultured on all four commercial culture-based mastitis tests and routine bacteriological milk culture. Of these samples, 671 had an SCC > 200,000 cells/mL or an abnormal milk appearance and were thus considered to originate from quarters that likely had mastitis. One hundred forty-one milk samples had an SCC ≤ 200,000 cells/mL (low SCC samples). Of 54 samples no SCC was measured, with most of these because the machine was broken. A total of 597 samples was read by two readers.

Most of the samples yielded one type of colonies (66%) according to routine bacteriological milk culture (Table 1). The most prevalent bacteria cultured with routine bacteriological milk culture were Gram-positive bacteria, followed by Gram-negative bacteria. From 16% of the milk samples no relevant mastitis-causing bacteria were cultured and were thus considered to have no growth. Most frequent cultured species were *E. coli*, *S. aureus*, CNS, and *S. uberis*. *Streptococcus agalactiae* was cultured least frequent. From 106 milk samples more than two different colony morphologies were cultured and thus were considered contaminated. The cross-tabulated results of the four commercial culture-based tests with the results of routine bacteriological culture of milk are shown in Table 3:

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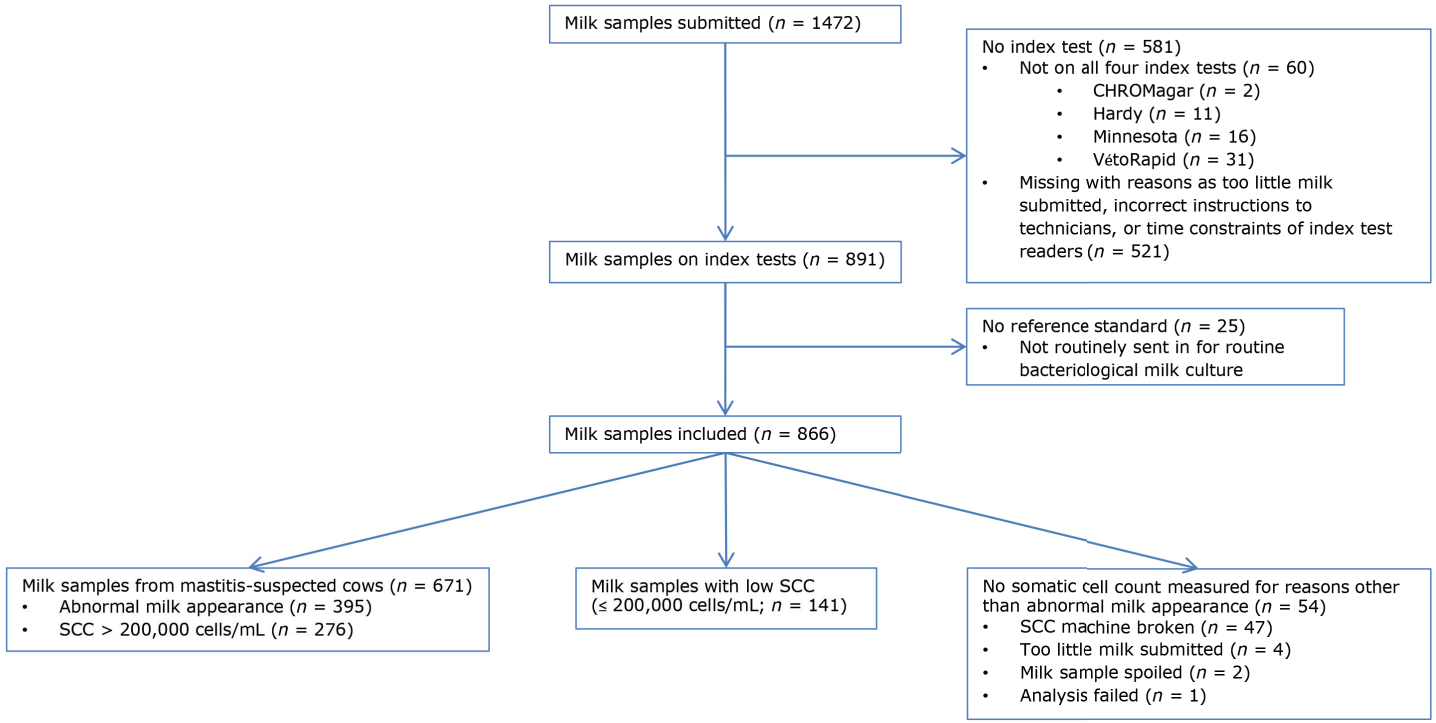

Figure 1. Flow diagram of milk samples submitted to the GD Animal Health service laboratory and used to assess agreement between four commercial culture-based tests (index tests) and routine bacteriological milk culture
The four commercial culture-based tests were:
CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate

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Table 2. Determination possibilities of routine bacteriological milk culture and of four commercial culture-based mastitis tests

	Bacteriological milk culture	CHROMagar[1]	Hardy[2]	Minnesota[3]	VétoRapid[4]
No growth	X	X	X	X	X
Gram-positive bacteria	X	X	X	X	X
Staphylococcus spp.	X		X	X	X
S. aureus	X	X	X	X	X
CNS	X				X
Streptococcus spp.	X		X	X	X
S. uberis	X	X	X		X
S. agalactiae	X	X	X	X	X
S. dysgalactiae	X				X
Enterococcus spp.	X				X
Other Gram-positive bacteria	X	X			
Gram-negative bacteria	X	X	X	X	X
Lactose fermenting bacteria	X		X		
Escherichia coli	X	X			X
KEC	X	X			X
Non-lactose fermenting bacteria	X		X		X
Pseudomonas spp.	X	X			
Other Gram-negative bacteria	X	X	X		X

[1] CHROMagar Mastitis (CHROMagar, Paris, France) consisting of two separate plates
[2] Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
[3] Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
[4] VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate

Just above 55% of the samples were cultured on the same day both by performing the routine procedure for bacteriological milk culture and the commercial culture-based tests. The remaining samples (*n* = 388) were stored at -20 °C to be cultured using the commercial culture-based mastitis tests at a later time within the study period. Reasons for storing samples at -20 °C were samples being submitted on days before leaves (weekend or holiday (*n* = 162)) or time constraints (*n* = 226).

The agreement beyond change between the four commercial culture-based tests and routine bacteriological milk culture is shown in Table 4. The agreement for Gram-positive bacteria was fair for all tests except for Hardy Diagnostics Mastitis Triplate

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that consistently had a significantly lower agreement (0.14 (95% CI 0.11-0.16) using all samples) than the other three tests. The overall low agreement for Gram-positive bacteria was caused by routine bacteriological culture of milk resulting in no growth (29% of disagreed results over all four tests together (318/1,086)), Gram-negative bacteria (34% of the disagreed results (370/1,086)), or contamination (36% of the disagreed results (387/1,086)), and by the commercial culture-based tests resulting in no growth (84% of the disagreed results over all tests together (179/214)), and Gram-negative bacteria (8% of the disagreed results (18/214)).

The agreement was substantial for Gram-negative bacteria ranging from 0.69 (95% CI 0.66-0.73) to 0.71 (95% CI 0.68-0.74). In low SCC samples the agreement was moderate ranging from 0.40 (95% CI 0.31-0.48) to 0.48 (95% CI 0.39-0.56). However, PABAK ranged from 0.72 (95% CI 0.57-0.87) to 0.80 (95% CI 0.67-0.93), which was comparable with the other sample types, indicating an effect of prevalence and bias on the calculated kappa value in low SCC samples.

Table 3. Cross-tabulation of four commercial culture-based mastitis tests with routine bacteriological milk culture to detect mastitis-causing pathogens in milk samples submitted for routine bacteriological milk culture

		Bacteriological culture of milk (*n* = 866)							
		Gram-positive		**Gram-negative**		**No growth**		**Contaminated**	
	Result	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
CHROMagar	+	333	250	205	82	48	71	39	74
	-	76	207	22	557	89	658	67	686
Hardy	+	376	314	197	77	31	33	52	130
	-	33	143	30	562	106	696	54	630
Minnesota	+	358	261	200	82	50	66	32	71
	-	51	196	27	557	87	663	74	689
VétoRapid	+	342	261	193	74	64	83	49	109
	-	67	196	34	565	73	646	57	651

CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate

The low agreement for no growth in all samples was caused by routine bacteriological milk culture resulting in Gram-positive bacteria (73% of the disagreed results over all four tests together (179/249)), Gram-negative bacteria (21% of the disagreed

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results (53/249)), or contamination (9% of the disagreed results (23/249)), and by the commercial culture-based tests indicating Gram-positive bacteria (90% of the disagreed results over all four tests together (318/352)), and Gram-negative bacteria (15% of the disagreed results (53/352)). For both no growth and contamination PABAK was moderate to substantial, and higher than the calculated kappa value, which was slight to fair.

The agreement beyond chance between two readers was 0.72 or higher for each commercial culture-based test for all diagnostic categories (Table 5). Contamination was the exception. For contamination in all samples and mastitis samples readers agreed most using CHROMagar Mastitis or VétoRapid (kappa ranging 0.61 (95% CI 0.57-0.65) to 0.67 (95% CI 0.63-0.71)) while the agreement was significantly lower using Hardy Diagnostics Mastitis Triplate or Minnesota Easy Culture System II Tri-plate (kappa ranging 0.31 (95% CI 0.27-0.35) to 0.40 (95% CI 0.36-0.43)). The agreement between readers was significantly lower for no growth in all samples using Minnesota Easy Culture System II Tri-plate, although kappa still was substantial (0.79 (95% CI 0.76-0.83). The same was seen in mastitis samples where Minnesota Easy Culture System II Tri-plate also had the lowest kappa (0.77 (95% CI 0.73-0.81). In low SCC samples all readers agreed almost perfectly regardless the commercial culture-based test used. Also for Gram-positive bacteria readers agreed almost perfectly using Minnesota Easy Culture System II Tri-plate or VétoRapid regardless sample type.

When the results of two commercial culture-based tests were compared, Minnesota Easy Culture System II Tri-plate, VétoRapid, CHROMagar Mastitis had all a significant higher agreement with each other for Gram-positive bacteria in all samples than CHROMagar Mastitis, Minnesota Easy Culture System II Tri-plate, or VétoRapid had with Hardy Diagnostics Mastitis Triplate (Table 6). All commercial culture-based tests had the same level of agreement with each other for Gram-negative bacteria, regardless sample type. The agreement of the commercial culture-based tests with each other was lower than the agreement between two readers reading the same commercial culture-based tests, but generally higher than the agreement of the commercial culture-based tests with routine bacteriological culture of milk.

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Table 4. Agreement of four commercial culture-based mastitis tests with routine bacteriological milk culture for four diagnostic categories using milk samples submitted for routine bacteriological milk culture. Three types of samples were compared: all submitted samples, samples originating from quarters suspected of having mastitis, and samples with SCC ≤ 200,000 cells/mL (low SCC samples)

	Gram-positive				Gram-negative				No growth				Contaminated			
	κ[1]	(95% CI)	PABAK[2]	(95% CI)	κ	(95% CI)	PABAK	(95% CI)	κ	(95% CI)	PABAK	(95% CI)	κ	(95% CI)	PABAK	(95% CI)
All samples (n = 866)																
CHROMagar	0.23[a]	(0.20,0.26)	0.25	(0.19,0.31)	0.71	(0.68,0.74)	0.76	(0.70,0.82)	0.26[a,b]	(0.23,0.29)	0.63	(0.56,0.69)	0.26	(0.23,0.29)	0.67	(0.61,0.74)
Hardy	0.14[b]	(0.11,0.16)	0.20	(0.15,0.25)	0.70	(0.67,0.73)	0.75	(0.69,0.81)	0.22[a]	(0.19,0.25)	0.68	(0.62,0.74)	0.23	(0.20,0.26)	0.57	(0.51,0.64)
Minnesota	0.25[a]	(0.22,0.28)	0.28	(0.22,0.34)	0.70	(0.67,0.73)	0.75	(0.69,0.81)	0.30[b,c]	(0.27,0.33)	0.65	(0.59,0.71)	0.21	(0.18,0.24)	0.66	(0.60,0.73)
VétoRapid	0.22[a]	(0.19,0.25)	0.24	(0.18,0.30)	0.69	(0.66,0.73)	0.75	(0.69,0.81)	0.34[c]	(0.31,0.38)	0.64	(0.58,0.70)	0.26	(0.23,0.28)	0.62	(0.55,0.68)
Mastitis samples (n = 671)																
CHROMagar	0.25[a]	(0.21,0.28)	0.25	(0.19,0.32)	0.74	(0.70,0.77)	0.77	(0.70,0.83)	0.24[a]	(0.21,0.27)	0.63	(0.55,0.70)	0.19	(0.16,0.22)	0.67	(0.60,0.74)
Hardy	0.15[b]	(0.12,0.18)	0.19	(0.13,0.25)	0.71	(0.68,0.75)	0.75	(0.68,0.81)	0.26[a,b]	(0.23,0.30)	0.70	(0.63,0.77)	0.18	(0.15,0.21)	0.55	(0.48,0.63)
Minnesota	0.25[a]	(0.22,0.28)	0.26	(0.19,0.33)	0.73	(0.69,0.76)	0.76	(0.70,0.82)	0.30[a,b]	(0.26,0.33)	0.65	(0.58,0.72)	0.17	(0.14,0.20)	0.67	(0.60,0.74)
VétoRapid	0.22[a]	(0.19,0.26)	0.24	(0.17,0.30)	0.72	(0.69,0.76)	0.76	(0.69,0.82)	0.32[b]	(0.29,0.36)	0.65	(0.57,0.72)	0.20	(0.17,0.23)	0.61	(0.54,0.69)
Low SCC samples (n = 141)																
CHROMagar	0.14[a]	(0.08,0.20)	0.23	(0.09,0.37)	0.41	(0.33,0.50)	0.73	(0.59,0.88)	0.33[a]	(0.25,0.41)	0.59	(0.43,0.75)	0.40	(0.32,0.48)	0.67	(0.52,0.83)
Hardy	-0.03[b]	(-0.05,0.00)	0.21	(0.07,0.34)	0.40	(0.32,0.48)	0.73	(0.59,0.88)	0.11[b]	(0.06,0.16)	0.60	(0.44,0.76)	0.33	(0.26,0.41)	0.59	(0.42,0.75)
Minnesota	0.16[a]	(0.10,0.22)	0.31	(0.15,0.46)	0.40	(0.31,0.48)	0.72	(0.57,0.87)	0.28[a]	(0.20,0.35)	0.61	(0.45,0.77)	0.26	(0.19,0.34)	0.64	(0.48,0.80)
VétoRapid	0.17[a]	(0.11,0.23)	0.26	(0.11,0.40)	0.48	(0.39,0.56)	0.80	(0.67,0.93)	0.41[a]	(0.33,0.49)	0.60	(0.44,0.76)	0.35	(0.27,0.43)	0.61	(0.45,0.77)

CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate
[a-c]Significant differences within columns per sample type
[1] Fleiss kappa accounting for varying identity of two readers with 95% CI
[2] PABAK = prevalence adjusted and bias adjusted kappa calculated as $2 * P_o - 1$, with $P_o = {}^{a+d}/_{n}$ with 95% CI

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Table 5. Agreement between readers of four commercial culture-based mastitis tests read by two readers for four diagnostic categories using milk samples submitted for routine bacteriological milk culture. Three groups of samples were compared: all submitted samples, samples originating from quarters suspected of having mastitis, and samples with SCC ≤ 200,000 cells/mL (low SCC samples)

	Gram-positive				Gram-negative				No growth				Contaminated			
	κ[1]	95% CI	PABAK[2]	95% CI	κ	95% CI	PABAK	95% CI	κ	95% CI	PABAK	95% CI	κ	95% CI	PABAK	95% CI
All samples (n = 597)																
CHROMagar	0.74[a]	(0.71,0.78)	0.78[a]	(0.72,0.83)	0.91	(0.89,0.94)	0.93	(0.90,0.96)	0.87[a,b]	(0.84,0.90)	0.94[a,b]	(0.91,0.97)	0.61[a]	(0.57,0.65)	0.83[a,c]	(0.78,0.87)
Hardy	0.72[a]	(0.68,0.75)	0.83[a,b]	(0.78,0.87)	0.87	(0.84,0.90)	0.89	(0.85,0.93)	0.86[a]	(0.83,0.88)	0.97[a]	(0.95,0.99)	0.40[b]	(0.36,0.43)	0.61[b]	(0.55,0.67)
Minnesota	0.83[b]	(0.80,0.86)	0.87[b]	(0.83,0.91)	0.89	(0.86,0.91)	0.91	(0.87,0.94)	0.79[b]	(0.76,0.83)	0.90[b]	(0.87,0.94)	0.31[c]	(0.27,0.35)	0.73[b,c]	(0.67,0.78)
VétoRapid	0.86[b]	(0.83,0.89)	0.88[b]	(0.85,0.92)	0.89	(0.87,0.92)	0.91	(0.88,0.94)	0.89[a,b]	(0.87,0.92)	0.94[a,b]	(0.91,0.97)	0.67[a]	(0.63,0.71)	0.80[c]	(0.75,0.85)
Mastitis samples (n = 454)																
CHROMagar	0.73[a,b]	(0.69,0.77)	0.76	(0.70,0.82)	0.92	(0.89,0.94)	0.93	(0.89,0.96)	0.88[a]	(0.85,0.91)	0.95[a,b]	(0.92,0.98)	0.64[a]	(0.59,0.68)	0.84[a]	(0.79,0.89)
Hardy	0.68[a]	(0.64,0.72)	0.79	(0.73,0.85)	0.88	(0.85,0.91)	0.89	(0.85,0.93)	0.83[a,b]	(0.80,0.87)	0.96[a]	(0.93,0.99)	0.36[a,b]	(0.31,0.40)	0.58[b]	(0.50,0.65)
Minnesota	0.81[b,c]	(0.77,0.84)	0.84	(0.79,0.89)	0.89	(0.87,0.92)	0.91	(0.87,0.95)	0.77[b]	(0.73,0.81)	0.89[b]	(0.85,0.93)	0.33[a,b]	(0.29,0.38)	0.73[a]	(0.67,0.79)
VétoRapid	0.84[c]	(0.80,0.87)	0.86	(0.82,0.91)	0.89	(0.86,0.92)	0.90	(0.86,0.94)	0.87[a]	(0.84,0.90)	0.93[a,b]	(0.90,0.96)	0.67[a]	(0.63,0.71)	0.80[a]	(0.75,0.86)
Low SCC samples (n = 106)																
CHROMagar	0.76[a]	(0.68,0.84)	0.81[a]	(0.70,0.92)	0.82	(0.75,0.89)	0.91	(0.82,0.99)	0.88[a]	(0.82,0.94)	0.92	(0.85,1.00)	0.53[a]	(0.44,0.63)	0.75	(0.63,0.88)
Hardy	1.00[b]	(1.00,1.00)	1.00[b]	(1.00,1.00)	0.77	(0.69,0.85)	0.87	(0.77,0.96)	1.00[b]	(1.00,1.00)	1.00	(1.00,1.00)	0.58[a]	(0.49,0.68)	0.74	(0.61,0.86)
Minnesota	0.93[c]	(0.88,0.98)	0.96[a,b]	(0.91,1.00)	0.82	(0.74,0.89)	0.89	(0.80,0.97)	0.91[a,c]	(0.86,0.97)	0.96	(0.91,1.00)	0.12[b]	(0.06,0.18)	0.70	(0.56,0.83)
VétoRapid	0.95[c]	(0.91,0.99)	0.96[a,b]	(0.91,1.00)	0.86	(0.80,0.93)	0.94	(0.88,1.00)	0.97[b,c]	(0.94,1.00)	0.98	(0.94,1.00)	0.67[a]	(0.58,0.76)	0.79	(0.68,0.91)

CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate
[a-c]Significant differences within columns per sample type
[1] Fleiss kappa accounting for varying identity of two readers with 95% CI
[2] PABAK = prevalence adjusted and bias adjusted kappa calculated as $2 * P_o - 1$, with $P_o = {}^{a+d}/_{n}$ with 95% CI

Minnesota Easy Culture System II Tri-plate had a significantly higher agreement with routine bacteriological culture of milk for *Staphylococcus* spp. (0.30 (95% CI 0.27-0.33)) than Hardy Diagnostics Mastitis Triplate (0.22 (95% CI 0.19-0.25)) and VétoRapid (0.24 (95% CI 0.21-0.26)) (Table 7). Also for *S. aureus* the agreement of Minnesota Easy Culture System II Tri-plate with routine bacteriological culture of milk was higher, as well as the agreement of VétoRapid with routine bacteriological culture of milk, than CHROMagar Mastitis and Hardy Diagnostics Mastitis Triplate. For Gram-negative bacteria as *E. coli* and the group KEC, the agreement of CHROMagar Mastitis and VétoRapid with routine bacteriological culture of milk were moderate to substantial, while PABAK was almost perfect. The effect of prevalence and bias on the kappa value became higher in low SCC samples.

Uncertainty reading results of commercial culture-based tests

The number of results readers were uncertain about was highest using CHROMagar Mastitis or Hardy Diagnostics Mastitis Triplate, both 30% of the results. These were followed by VétoRapid with 18% of the results leading to uncertainty, and Minnesota Easy Culture System II Tri-plate with 14%. When the reader noted to be uncertain, most often a Gram-positive result (> 90%) was noted by the reader, regardless the commercial culture-based test used.

Discussion

To minimize unnecessary antimicrobial use in the dairy industry, there is a need for tests to determine the IMI status of a cow. Bacteriological milk culture could be used, although the definition of the IMI status based on bacteriological milk culture results has been debated for many years (Bradley et al., 2005; Lam et al., 2009; Andersen et al., 2010; Dohoo et al., 2011a). Currently, the most common test used to determine an IMI is bacteriological culture of milk. However, the sensitivity and specificity of bacteriological culture of milk to determine an IMI is low (Dohoo et al., 2011b). Nevertheless, a commercial culture-based test might be a practical tool to facilitate prudent use of antimicrobials on dairy farms (Ruegg et al., 2009; Wallace, 2011; Keefe et al., 2013).

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Table 6. Agreement of four commercial culture-based mastitis tests when compared to each other for four diagnostic categories using milk samples submitted for routine bacteriological milk culture. Three types of samples were compared: all submitted samples, samples originating from quarters suspected of having mastitis, and samples with SCC ≤ 200,000 cells/mL (low SCC samples)

		Gram-positive				Gram-negative			
		κ[1]	(95% CI)	PABAK[2]	(95% CI)	κ	(95% CI)	PABAK	(95% CI)
All samples (n = 866)									
Hardy	CHROMagar	0.42[a,c]	(0.39,0.45)	0.55	(0.48,0.61)	0.82	(0.79,0.84)	0.84	(0.79,0.89)
	Minnesota	0.43[a,c]	(0.40,0.47)	0.58	(0.52,0.65)	0.78	(0.75,0.81)	0.81	(0.75,0.86)
	VétoRapid	0.38[a]	(0.35,0.41)	0.53	(0.47,0.60)	0.77	(0.74,0.80)	0.80	(0.75,0.85)
Minnesota	CHROMagar	0.51[b]	(0.48,0.55)	0.59	(0.52,0.65)	0.79	(0.77,0.82)	0.82	(0.77,0.87)
	VétoRapid	0.49[b,c]	(0.45,0.52)	0.57	(0.51,0.64)	0.77	(0.74,0.80)	0.80	(0.75,0.86)
CHROMagar	VétoRapid	0.49[b,c]	(0.45,0.52)	0.56	(0.49,0.62)	0.77	(0.74,0.80)	0.80	(0.75,0.85)
Mastitis samples (n = 671)									
Hardy	CHROMagar	0.41[a]	(0.37,0.45)	0.52	(0.44,0.59)	0.81	(0.78,0.84)	0.83	(0.77,0.89)
	Minnesota	0.42[a,c]	(0.39,0.46)	0.54	(0.47,0.62)	0.78	(0.75,0.81)	0.80	(0.74,0.86)
	VétoRapid	0.37[a]	(0.34,0.41)	0.51	(0.43,0.58)	0.78	(0.75,0.81)	0.80	(0.74,0.86)
Minnesota	CHROMagar	0.51[b]	(0.47,0.55)	0.56	(0.49,0.64)	0.80	(0.77,0.83)	0.82	(0.76,0.87)
	VétoRapid	0.49[b,c]	(0.45,0.53)	0.56	(0.49,0.64)	0.78	(0.75,0.81)	0.80	(0.74,0.86)
CHROMagar	VétoRapid	0.50[b,c]	(0.46,0.53)	0.55	(0.48,0.63)	0.79	(0.76,0.82)	0.81	(0.75,0.87)
Low SCC samples (n = 141)									
Hardy	CHROMagar	0.31[a]	(0.23,0.38)	0.60	(0.44,0.76)	0.71	(0.63,0.78)	0.82	(0.69,0.95)
	Minnesota	0.30[a]	(0.22,0.37)	0.68	(0.53,0.84)	0.71	(0.63,0.78)	0.82	(0.69,0.95)
	VétoRapid	0.32[a]	(0.24,0.39)	0.60	(0.44,0.76)	0.65	(0.58,0.73)	0.81	(0.68,0.94)
Minnesota	CHROMagar	0.40[a,b]	(0.32,0.48)	0.61	(0.45,0.77)	0.73	(0.66,0.81)	0.83	(0.71,0.96)
	VétoRapid	0.41[a,b]	(0.32,0.49)	0.61	(0.45,0.77)	0.67	(0.59,0.75)	0.81	(0.69,0.94)
CHROMagar	VétoRapid	0.50[b]	(0.42,0.58)	0.62	(0.46,0.78)	0.65	(0.57,0.73)	0.81	(0.68,0.94)

CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate
[a-c]Significant differences within columns per sample type
[1] Fleiss kappa accounting for varying identity of two readers with 95% CI
[2] PABAK = prevalence adjusted and bias adjusted kappa calculated as $2 * P_o - 1$, with $P_o = {}^{a+d}/_{n}$ with 95% CI

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	No growth				Contaminated			
	κ	(95% CI)	PABAK	(95% CI)	κ	(95% CI)	PABAK	(95% CI)
	0.49	(0.45,0.52)	0.81	(0.75,0.86)	0.30[a]	(0.27,0.33)	0.60	(0.54,0.67)
	0.43	(0.40,0.47)	0.79	(0.73,0.84)	0.27[a]	(0.24,0.30)	0.60	(0.53,0.67)
	0.46	(0.43,0.50)	0.75	(0.70,0.81)	0.33[a]	(0.29,0.36)	0.58	(0.51,0.64)
	0.50	(0.46,0.53)	0.76	(0.71,0.82)	0.29[a]	(0.26,0.32)	0.69	(0.63,0.75)
	0.44	(0.40,0.47)	0.71	(0.65,0.77)	0.32[a]	(0.29,0.35)	0.65	(0.59,0.71)
	0.50	(0.47,0.53)	0.74	(0.68,0.80)	0.40[b]	(0.37,0.43)	0.68	(0.62,0.75)
	0.52	(0.49,0.56)	0.82	(0.76,0.88)	0.30[a]	(0.26,0.33)	0.60	(0.53,0.67)
	0.46	(0.43,0.50)	0.79	(0.73,0.85)	0.24[a]	(0.21,0.28)	0.58	(0.51,0.66)
	0.51	(0.47,0.54)	0.77	(0.70,0.83)	0.31[a,b]	(0.28,0.35)	0.57	(0.49,0.64)
	0.49	(0.45,0.53)	0.76	(0.69,0.82)	0.26[a]	(0.23,0.30)	0.69	(0.62,0.76)
	0.45	(0.42,0.49)	0.72	(0.65,0.79)	0.32[a,c]	(0.28,0.35)	0.67	(0.60,0.74)
	0.51	(0.47,0.55)	0.75	(0.69,0.82)	0.38[b]	(0.34,0.42)	0.69	(0.62,0.76)
	0.28[a]	(0.21,0.35)	0.70	(0.55,0.85)	0.35[a,c]	(0.27,0.42)	0.60	(0.44,0.76)
	0.24[a]	(0.17,0.32)	0.75	(0.61,0.89)	0.18[b]	(0.12,0.25)	0.55	(0.38,0.71)
	0.29[a]	(0.21,0.36)	0.65	(0.49,0.81)	0.35[a,c]	(0.27,0.43)	0.58	(0.41,0.74)
	0.51[b]	(0.43,0.60)	0.75	(0.61,0.89)	0.36[a,c]	(0.28,0.44)	0.69	(0.54,0.85)
	0.39[a,b]	(0.31,0.47)	0.64	(0.48,0.80)	0.25[a,b]	(0.18,0.32)	0.61	(0.44,0.77)
	0.48[b]	(0.40,0.56)	0.66	(0.50,0.82)	0.48[c]	(0.40,0.56)	0.70	(0.54,0.85)

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Table 7. Agreement of four commercial culture-based mastitis tests with routine bacteriological milk culture for mastitis-causing pathogens using milk samples submitted for routine bacteriological milk culture. Three types of samples were compared: all submitted samples, samples originating from quarters suspected of having mastitis, and samples with SCC ≤ 200,000 cells/mL (low SCC samples)

	CHROMagar				Hardy	
	κ[1]	(95% CI)	PABAK[2]	(95% CI)	κ	(95% CI)
All samples (*n* = 866)						
Staphylococcus spp.	-	-	-	-	0.22[a]	(0.19, 0.25)
S. aureus	0.33[a]	(0.30, 0.36)	0.64	(0.57,0.70)	0.34[a]	(0.30, 0.37)
CNS	-	-	-	-	-	-
Streptococcus spp.	-0.24[a]	(-0.27,-0.20)	0.32[A]	(0.25,0.38)	0.12[b]	(0.10, 0.14)
S. uberis	0.09[a]	(0.07, 0.11)	0.42	(0.35,0.49)	-	-
S. agalactiae	-0.12[a]	(-0.15,-0.10)	0.56[A]	(0.49,0.63)	-0.06[b]	(-0.08,-0.05)
S. dysgalactiae	-	-	-	-	-	-
Lactose-fermenting bacteria	-	-	-	-	0.29	(0.26, 0.32)
Escherichia coli	0.75	(0.72, 0.78)	0.84	(0.79,0.89)	-	-
KEC	0.56	(0.53, 0.59)	0.84	(0.79,0.89)	-	-
Non-lactose-fermenting bacteria	-	-	-	-	0.10	(0.08, 0.12)
Pseudomonas spp.	0.04	(0.03, 0.05)	0.95	(0.92,0.98)	-	-
Mastitis samples (*n* = 671)						
Staphylococcus spp.	-	-	-	-	0.20[a]	(0.17, 0.23)
S. aureus	0.36[a,b]	(0.33, 0.40)	0.67	(0.60,0.74)	0.33[a]	(0.30, 0.37)
CNS	-	-	-	-	-	-
Streptococcus spp.	-0.24[a]	(-0.28,-0.20)	0.33[A,B]	(0.26,0.40)	0.15[b]	(0.13, 0.18)
S. uberis	0.12[a]	(0.09, 0.14)	0.43	(0.35,0.50)	-	-
S. agalactiae	-0.11[a]	(-0.14,-0.09)	0.59[A]	(0.52,0.67)	-0.06[b]	(-0.08,-0.04)
S. dysgalactiae	-	-	-	-	-	-
Lactose-fermenting bacteria	-	-	-	-	0.26	(0.23, 0.30)
E. coli	0.76	(0.73, 0.80)	0.84	(0.78,0.89)	-	-
KEC	0.58	(0.54, 0.62)	0.84	(0.78,0.89)	-	-
Non-lactose-fermenting bacteria	-	-	-	-	0.11[a]	(0.09, 0.14)
Pseudomonas spp.	0.07	(0.05, 0.09)	0.95	(0.92,0.98)	-	-
Low SCC samples (*n* = 141)						
Staphylococcus spp.	-	-	-	-	0.17[a]	(0.11, 0.24)
S. aureus	0.24[a]	(0.17, 0.31)	0.52	(0.36,0.69)	0.44[b,c]	(0.36, 0.52)
CNS	-	-	-	-	-	-
Streptococcus spp.	-0.21[a]	(-0.28,-0.14)	0.26[A,B]	(0.12,0.41)	0.06[b]	(0.02, 0.10)
S. uberis	0.04[a]	(0.01, 0.07)	0.32	(0.17,0.48)	-	-
S. agalactiae	-0.12[a]	(-0.18,-0.06)	0.56[A]	(0.40,0.72)	-0.07[a,b]	(-0.12,-0.03)
S. dysgalactiae	-	-	-	-	-	-
Lactose-fermenting bacteria	-	-	-	-	0.38	(0.30, 0.46)
E. coli	0.45	(0.36, 0.53)	0.84	(0.72,0.96)	-	-
KEC	0.27	(0.19, 0.34)	0.87	(0.76,0.98)	-	-
Non-lactose-fermenting bacteria	-	-	-	-	-0.03	(-0.05, 0.00)
Pseudomonas spp.	-0.02	(-0.04,0.01)	0.88	(0.78,0.99)	-	-

CHROMagar Mastitis (CHROMagar, Paris, France), a combination of two separate plates
Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, Santa Maria, CA, USA), a triplate
Minnesota Easy Culture System II Tri-plate (Minnesota University, St. Paul, MN, USA), a triplate
VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands), a triplate

Hardy			Minnesota				VétoRapid		
PABAK	(95% CI)	κ	(95% CI)	PABAK	(95% CI)	κ	(95% CI)	PABAK	(95% CI)
0.30	(0.24,0.36)	0.30^{b}	(0.27,0.33)	0.39	(0.32,0.45)	0.24^{a}	(0.21,0.26)	0.29	(0.23,0.35)
0.66	(0.59,0.72)	0.47^{b}	(0.43,0.50)	0.69	(0.63,0.76)	0.40^{b}	(0.37,0.44)	0.62	(0.55,0.68)
-	-	-	-	-	-	0.05	(0.04,0.07)	0.35	(0.29,0.42)
0.19^{B}	(0.13,0.24)	0.28^{c}	(0.25,0.31)	0.40^{A}	(0.33,0.46)	0.28^{c}	(0.25,0.31)	0.39^{A}	(0.32,0.45)
-	-	-	-	-	-	0.22^{b}	(0.19,0.25)	0.52	(0.45,0.58)
0.77^{B}	(0.71,0.82)	0.00^{c}	(-0.01,0.00)	0.99^{C}	(0.98,1.00)	-0.01^{c}	(-0.01,0.00)	0.98^{C}	(0.96,1.00)
-	-	-	-	-	-	0.28	(0.25,0.31)	0.84	(0.80,0.89)
0.62	(0.56,0.68)	-	-	-	-	-	-	-	-
-	-	-	-	-	-	0.72	(0.69,0.75)	0.84	(0.79,0.89)
-	-	-	-	-	-	0.53	(0.50,0.57)	0.83	(0.78,0.88)
0.88	(0.84,0.93)	-	-	-	-	0.13	(0.11,0.16)	0.92	(0.77,1.00)
-	-	-	-	-	-	-	-	-	-
0.29	(0.23,0.36)	0.27^{b}	(0.23,0.30)	0.37	(0.30,0.45)	$0.25^{a,b}$	(0.21,0.28)	0.31	(0.24,0.38)
0.67	(0.60,0.74)	0.44^{b}	(0.40,0.47)	0.69	(0.62,0.76)	0.42^{b}	(0.38,0.45)	0.63	(0.56,0.71)
-	-	-	-	-	-	0.05	(0.03,0.06)	0.39	(0.31,0.46)
0.23^{A}	(0.16,0.29)	0.30^{c}	(0.27,0.34)	0.42^{B}	(0.35,0.50)	0.29^{c}	(0.26,0.32)	0.40^{B}	(0.32,0.47)
-	-	-	-	-	-	0.25^{b}	(0.22,0.28)	0.54	(0.47,0.62)
0.78^{B}	(0.72,0.84)	0.00^{c}	(-0.01,0.00)	0.99^{C}	(0.98,1.00)	-0.01^{c}	(-0.01,0.00)	0.98^{C}	(0.96,1.00)
-	-	-	-	-	-	0.27	(0.24,0.31)	0.85	(0.79,0.90)
0.57	(0.50,0.65)	-	-	-	-	-	-	-	-
-	-	-	-	-	-	0.73	(0.70,0.77)	0.83	(0.77,0.89)
-	-	-	-	-	-	0.58	(0.54,0.61)	0.83	(0.77,0.88)
0.88	(0.83,0.93)	-	-	-	-	0.17^{b}	(0.14,0.20)	0.92	(0.88,0.96)
-	-	-	-	-	-	-	-	-	-
0.19	(0.06,0.32)	0.31^{b}	(0.24,0.39)	0.33	(0.18,0.49)	$0.21^{a,b}$	(0.14,0.28)	0.22	(0.08,0.35)
0.64	(0.48,0.80)	0.58^{b}	(0.50,0.66)	0.72	(0.57,0.87)	$0.38^{a,c}$	(0.30,0.46)	0.55	(0.38,0.71)
-	-	-	-	-	-	0.10	(0.05,0.15)	0.27	(0.12,0.41)
0.10^{A}	(0.00,0.19)	0.28^{c}	(0.20,0.35)	0.35^{B}	(0.20,0.51)	0.32^{c}	(0.25,0.40)	0.42^{B}	(0.26,0.58)
-	-	-	-	-	-	0.20^{b}	(0.14,0.27)	0.49	(0.32,0.65)
0.70^{A}	(0.55,0.85)	0.00^{b}	(-0.01,0.01)	0.96^{B}	(0.89,1.00)	0.00^{b}	(-0.01,0.01)	0.96^{B}	(0.89,1.00)
-	-	-	-	-	-	0.32	(0.24,0.40)	0.89	(0.79,0.99)
0.86	(0.74,0.97)	-	-	-	-	-	-	-	-
-	-	-	-	-	-	0.58	(0.50,0.66)	0.88	(0.77,0.99)
-	-	-	-	-	-	0.17	(0.11,0.24)	0.89	(0.78,0.99)
0.80	(0.67,0.94)	-	-	-	-	-0.01	(-0.03,0.01)	0.96	(0.90,1.00)
-	-	-	-	-	-	-	-	-	-

[a-c] Significant differences within rows per sample type for kappa

[A-C] Significant difference within rows per sample type for PABAK

[1] Fleiss kappa accounting for varying identity of two readers with 95% CI

[2] PABAK = prevalence adjusted and bias adjusted kappa calculated as $2 * P_o - 1$, with $P_o = {}^{a+d}/_{n}$ with 95% CI

Earlier work showed that commercial culture-based tests are able to categorize mastitis cases into treatment groups (McCarron et al., 2009; Lago et al., 2011a; Cameron et al., 2013; Royster et al., 2014; Viora et al., 2014; Ganda et al., 2016). For Gram-negative bacteria we found an average agreement between commercial culture-based tests and routine bacteriological culture of milk of 0.70 (95% CI ranged 0.66-0.74), which was slightly lower than using Accumast (0.84 (95% CI 0.77-0.91)) (Ganda et al., 2016), but comparable with what was found using Minnesota Easy Culture System II Tri-plate (ranging 0.63-0.75 (95% CI ranging 0.57-0.81)) (Royster et al., 2014). However, the other diagnostic categories showed a lower agreement between commercial culture-based tests and routine bacteriological culture of milk than earlier found in literature. Especially for Gram-positive bacteria (kappa on average 0.21 (95% CI ranging 0.11-0.28)), and for no growth (kappa on average 0.28 (95% CI ranging 0.19-0.38)) kappa was lower than the earlier found 0.6 (Royster et al., 2014). Also the proportion of observed agreement ((total of agreed positive results + total of agreed negative results)/total results, also referred to as accuracy) for Gram-positive bacteria of 0.62 (95% CI 0.59-0.65) found in our study (data not shown) was lower than found using the Minnesota Easy Culture System II Tri-plate (0.81) (Ferreira et al., 2018), or using Petrifilm plates (0.85) (Mansion-de Vries et al., 2014). Explanations for this low agreement might be that routine bacteriological milk culture presented only relevant growth and assigned samples with irrelevant growth to the no growth or contamination category, while readers of commercial culture-based tests noted all growth present regardless the type of growth. Also, readers might have missed contamination, which was also noticed by Royster et al. (2014). However, when these commercial culture-based tests will be used on farms, both will result in cows being treated with the potential that treating is unnecessary (Ruegg et al., 2009; Lago et al., 2011a). Of bigger concern might be that approximately 5% of all samples cultured will be left untreated based on the no growth result of the commercial culture-based test while routine bacteriological milk culture resulted in Gram-positive bacteria, as Gram-positives should be treated (Ruegg et al., 2009). However, farmers probably tend to include more information to decide on treatment than solely a test result (Scherpenzeel et al., 2016) as 33% of the farmers decided to treat cows with a no growth result on commercial culture-based tests (Neeser et al., 2006). Therefore, the number of untreated cows that ideally ought to be treated likely will be limited.

The proportions of observed agreement we found for Gram-negative bacteria of on average 0.88 (95% CI 0.80-0.95), for no growth of on average 0.82 (95% CI 0.69-0.96),

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and for contamination of on average 0.82 (95% CI 0.73-0.90) (data not shown) were comparable with literature. For example to detect mastitis-associated bacteria using Accumast the proportion of observed agreement was 0.85 (Ganda et al., 2016) or 0.90, using Minnesota Easy Culture System II Tri-plate the observed agreement was 0.73, using SSGN it was 0.79, or using SSGNC it was 0.75 (Ferreira et al., 2018). These were also in line with the observed agreements found using Minnesota Easy Culture System II Bi-plate and Tri-plate (Royster et al., 2014).

A kappa paradox was quite explicit present for the categories no growth, and contamination as the observed agreement was high in these categories with many samples being negative for no growth or contamination according to both commercial culture-based tests and routine bacteriological milk culture. However, the kappa statistic was low. Interpreting solely the kappa statistic might lead to a paradoxical underestimation of the true agreement as prevalence and bias may have affected kappa estimates (Byrt et al., 1993). Calculating PABAK could give an estimation of that effect. In our study the kappa for no growth was on average 0.28 (95% CI ranged from 0.19-0.38) while PABAK was on average 0.65 (95% CI ranged from 0.56-0.74), and for contamination kappa was 0.24 (95% CI ranged from 0.18-0.29) while PABAK was on average 0.63 (95% CI ranged from 0.51-0.74). These differences in prevalence and bias among studies make direct comparison of kappa values difficult, especially when no contingency tables or additional measures like observed agreement or PABAK are given, and differences among studies should therefore be interpret with caution.

Different readers had a high agreement for Gram-positive bacteria, and even higher for Gram-negative bacteria, or no growth when reading the same commercial culture-based test, indicating that commercial culture-based tests were read consistently. For CHROMagar Mastitis and Hardy Diagnostics Mastitis Triplate the agreement for Gram-positive bacteria was lower than for Minnesota Easy Culture System II Tri-plate or VétoRapid which could be explained by readers being uncertain about their results more frequent using CHROMagar Mastitis and Hardy Diagnostics Mastitis Triplate. The same uncertainty likely caused the lower agreement for contamination too. Probably some readers noted Gram-positive bacteria but were uncertain about their decisions, while others noted contamination. That the agreement between readers was higher than between commercial culture-based tests and routine bacteriological milk culture was also seen in an earlier evaluation of the commercial culture-based test Minnesota Easy Culture System II Tri-plate (Royster et al., 2014).

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Within udder health, three types of cows might be considered for treatment: cows with clinical mastitis, with subclinical mastitis, and cows before drying off (Sears and McCarthy, 2003; van den Borne et al., 2010; Cameron et al., 2014; Krömker and Leimbach, 2017) and were considered the target populations for the commercial culture-based tests. Unfortunately, clinical information on the cows the milk samples were collected from lacked in our study. Therefore, we divided the samples in mastitis suspected samples and low SCC samples based on the SCC and milk appearance to examine the performance of the commercial culture-based tests among the different target populations. This differentiation could also be made by farmers using the Californian Mastitis Test and milk appearance (Dingwell et al., 2003; Bhutto et al., 2012). The performance of commercial culture-based tests among low SCC is for example of interest at drying off and previously commercial culture-based tests were able to detect IMI's within this group (Cameron et al., 2013). The most striking difference between the sample types was the agreement for Gram-negative bacteria that dropped from almost perfect using all samples or mastitis samples to moderate using low SCC samples. However, as also shown by PABAK, this agreement seemed to be underestimated as PABAK was comparable with the other sample types, likely explained by Gram-negative bacteria being less prevalent in low SCC samples. The study results might be biased as the source population likely was a subset of the target population, with an overrepresentation of more severe cases. Farmers might have submitted samples of, for example, more severe mastitis cases while with commercial culture-based tests available on farm they might use the test for all cases where a treatment decision needs to be made with respect to udder health. However, as the PABAK hardly differ among the different sample types, we consider it reasonable to extrapolate the results to the target population.

Veterinarians generally advise dairy farmers to culture milk samples on a regular basis to be able to manage udder health, either by using a commercial culture-based test on farm to decide on treatment, or by regularly submitting milk samples to a laboratory to be informed on the most important type of mastitis pathogens and antimicrobial susceptibility on farms (Barkema et al., 2006; Lam et al., 2009; Ruegg et al., 2009). Currently, roughly 72% of the cows with clinical mastitis is treated with antimicrobials in the Netherlands (Santman-Berends et al., 2015). Apparently, farmers decide on selective treatment using parameters other than test results since these test results are infrequently used. However, farmers do have a need to substantiate their decisions by additional testing (Griffioen et al., 2016). Commercial culture-based tests might be

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suitable to support these decisions. Although we found only for Gram-negative bacteria a good agreement between commercial culture-based tests and routine bacteriological culture of milk, and further on-farm evaluations are needed to determine the effect of these findings on udder health, commercial culture-based tests are of added value to support decisions whether and how to treat cows with intramammary infections.

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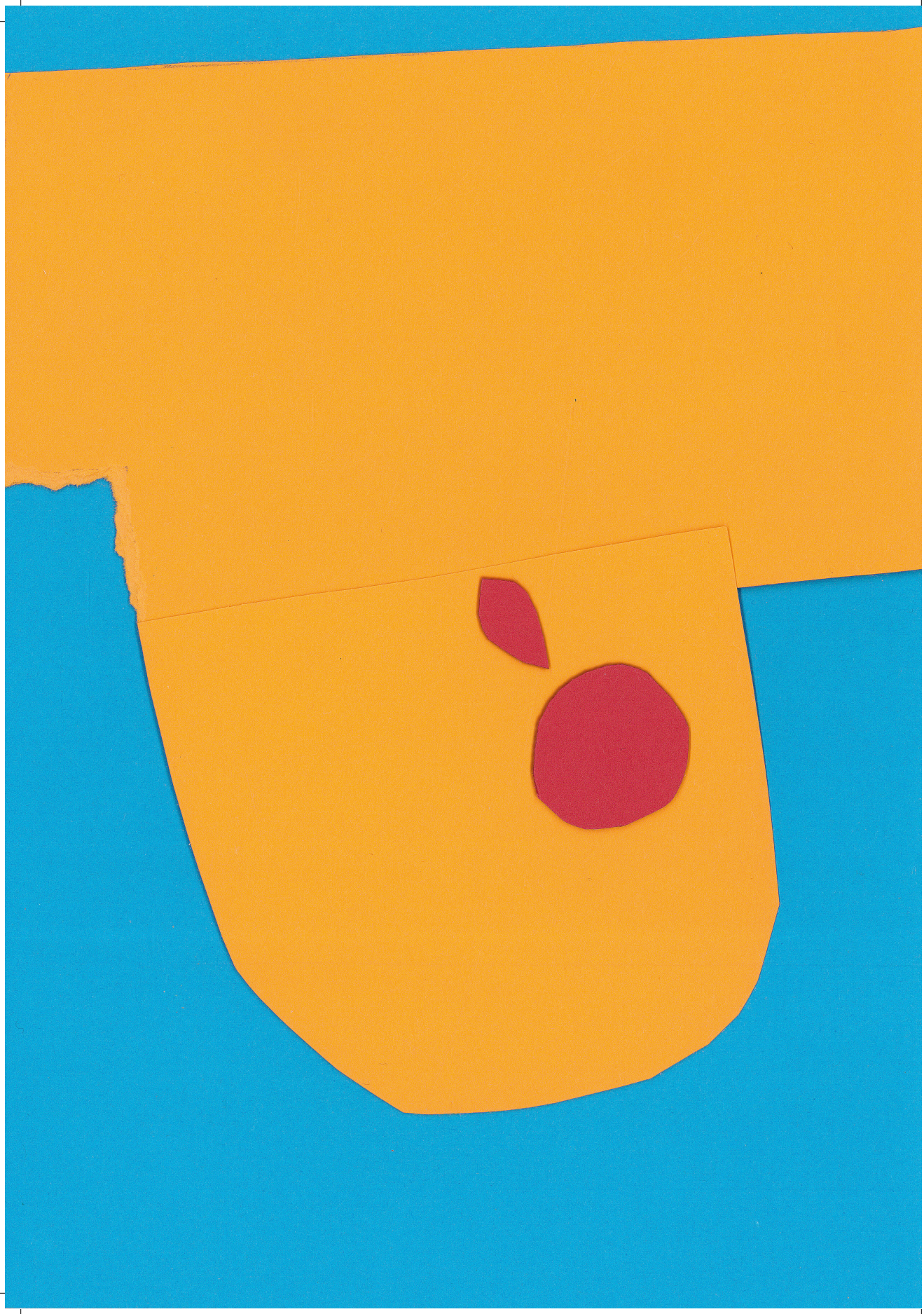

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4

Development and evaluation of four loop-mediated isothermal amplification assays to detect mastitis-causing bacteria in bovine milk samples

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Abstract

Currently available diagnostic tools are hardly used to decide on clinical mastitis treatments, because they do not fulfil farmers requirements, who prefer fast, sensitive, and on-site tests. Genotypic tests that do not require a growth step may be suitable for on-site testing, for example loop-mediated isothermal amplification (LAMP), that has been described as a sensitive test can be used on-site. Therefore, this study aimed to develop and evaluate LAMP assays for the detection of a subset of mastitis-causing pathogens, being *Escherichia coli*, *Klebsiella pneumonia, Staphylococcus aureus*, and *Streptococcus* spp. in milk from cows with clinical mastitis. Furthermore, a generic nucleic acid lateral flow immunoassay (NALFIA) was evaluated as a potential on-site readout of the LAMP assays. For each assay of LAMP and NALFIA, the limit of detection and analytical specificity was determined using well-characterized isolates, the diagnostic specificity was determined using selected (characterized) samples. In addition, the diagnostic specificity of LAMP was determined using blindly tested field samples. Bacteriological culture with identification by mass spectrometry was used as a reference method. The four assays had a kappa ≥ 0.73 with the reference method when testing the selected characterized samples, but lower kappa's when blindly testing field samples. After correcting for prevalence, kappa was ≥ 0.80 for the *E. coli*, *K. pneumoniae* and *S. aureus* assays. The *Streptococcus* spp. assay had a kappa of 0.47 with the reference method, probably caused by the assay broadly targeting a genus instead of a particular species. The NALFIA readout was found to have kappa ≥ 0.81 for the *E. coli*, *S. aureus*, and *Streptococcus* spp. assays at a generic runtime, but for the *K. pneumoniae* assay a shorter runtime could be used.

In conclusion, LAMP is a promising method for fast on-site tests for mastitis-causing pathogens if the current elaborate method for sample preparation would be replaced by a simplified protocol. The NALFIA is an easy and reliable readout for on-site use, with the remark that for the current assay designs a generic runtime is not yet possible for the chosen set of pathogens. If associated with a simple and fast sample preparation protocol, the combination of LAMP and NALFIA has the potential to enable fast and reliable on-site testing of clinical mastitis milk samples.

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Introduction

Mastitis is a common disease on dairies and has the highest contribution to the usage of antimicrobials in dairy cows (Pol and Ruegg, 2007; Kuipers et al., 2015). Currently, the decision to treat clinical mastitis with antimicrobials is mostly based on clinical signs (Sears and McCarthy, 2003; Griffioen et al., 2016), regardless of the actual cause. To enable targeted treatments, rapid and specific diagnostic tests to identify the cause of mastitis would be of added value.

Two types of diagnostic tests are frequently used for pathogen identification in mastitis: phenotypic and genotypic (Duarte et al., 2015). The advantage of genotypic tests over phenotypic tests is that the first have a short time to result, provided that the test can be applied directly on the sample and no growth step is necessary. Furthermore, genotypic tests are more sensitive than (growth-dependent) phenotypic tests, as they have the ability to detect DNA of dead, or less viable, pathogens (Lam et al., 2009; Duarte et al., 2015). Genotypic tests such as real-time PCR are used in diagnostic laboratories to detect mastitis pathogens, among other pathogens (Koskinen et al., 2009; Koskinen et al., 2010; Shome et al., 2011; Mahmmod et al., 2013a), but are not frequently used in practice for that purpose (Griffioen et al., 2016). Dutch farmers indicated that they are willing to use diagnostic tests more often if a simple test would be accessible close by, rather than at a distant laboratory (Griffioen et al., 2016). As expensive equipment is required to perform real-time PCR, these kinds of genotypic tests are seldomly available nearby a farm and thus time is required for transport of samples to a diagnostic laboratory. Thus, even though available real-time PCR tests are quick, the time to result is too long to comply with the farmers demands for treatment decisions. Therefore, less cumbersome options than real-time PCR, that can be performed at a veterinary clinic or on-farm, may fulfill the need of dairy farmers and may support incorporating diagnostic information into the decision-making process of mastitis treatments.

A technique that is advocated for on-site DNA amplification to detect pathogens, is loop-mediated isothermal amplification (LAMP) (Mori and Notomi, 2009; Ashraf and Imran, 2018). This technique has the advantage over (real-time) PCR in that expensive equipment for temperature cycling and detection of results is not required, and that the conditions for the extraction and purification of DNA are less demanding (Kaneko et al., 2007; Sowmya et al., 2012). These advantages have resulted in LAMP tests for on-site use to detect *Staphylococcus aureus* in food samples (Tian et al., 2018), and Bergomovirusses under field conditions, using a portable device (Tian et al., 2018; Wilisiani et al., 2019).

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A number of studies describe the ability of LAMP to detect pathogens in milk samples (Song et al., 2012; Yang et al., 2014; Sathish et al., 2016; Appelt et al., 2019; Sange et al., 2019). Numerous LAMP assays, for example those developed to detect *S. aureus* and *Streptococcus uberis*, have a time to result of only 1 to 2 hours and a high diagnostic accuracy (Tie et al., 2012; Cornelissen et al., 2016; Sheet et al., 2016). Thus, LAMP seems a promising method for on-site detection of mastitis-causing pathogens, and has the potential to fulfill the need of dairy farmers to obtain results within a short time. Therefore, this study aimed to develop and evaluate LAMP assays to detect a subset of mastitis-causing pathogens (*Escherichia coli*, *Klebsiella pneumonia, S. aureus*, and *Streptococcus* spp.) in milk from cows with clinical mastitis, and to evaluate a generic nucleic acid lateral flow immunoassay (NALFIA) as a potential on-site readout of LAMP.

Materials and methods

LAMP assay design

Four new LAMP assays were designed targeting genes described in literature as valid targets for detection of *E. coli*: *PhoA* gene (Shome et al., 2011), *K. pneumoniae*: *UreD* gene (Zamani et al., 2013), *S. aureus*: *nuc* gene (Tie et al., 2012*)*, and *Streptococcus* spp.: *16S rRNA* gene (Wang and Li, 2015). The gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) (accession numbers FJ546461, L07039, EF529606, and AP011114.1, respectively). Conserved regions were identified by sequence alignments using NBCI Blast search (http://blast.ncbi.nlm.nih.gov), and were used for primer design with LAMP Designer 1.14 (Premier Biosoft, Palo Alto, CA, USA). All LAMP assays were designed with six primers: two outer primers, two inner primers, and two loop primers (Table 1).

Evaluation of LAMP assays

The evaluation process of the four LAMP assays comprised 4 steps. First, the limit of detection was determined using dilution series of one isolate of the target bacterium. Second, the analytical specificity was determined using a fixed set of well characterized isolates of different bacterial species. Thereafter, the diagnostic specificity was determined using four sets of selected characterized milk samples, each compiled to allow evaluation of a specific LAMP assay. The last step was to determine the diagnostic performance by blindly testing 163 milk samples originating from cows with clinical mastitis collected in a field study for this purpose.

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Table 1. Sequences of four designed loop-mediated isothermal amplification (LAMP) primers

LAMP assay	Gene	Primer	Sequence (5'-3')
Escherichia coli	*PhoA*[1]	Ecoli 4 F3	TGT CAT TAC GTT GCG GAT T
		Ecoli 4 B3	CTT TGC TGA AAC GGC AAC
		Ecoli 4 FIP[5]	CTG ACG GCA ATA TGC CAG TGA TCG ATA TTG CCA TGG TAC G
		Ecoli 4 BIP[6]	ATT CGC TTC CGT CAC CGA GCG TGG TTA TCA GTT GGT
		Ecoli 4 Floop	GCT GGC TAG GAC CGA AAG
		Ecoli 4 Bloop	TTC AGT GAG GCA GCA TCG
Klebsiella pneumoniae	*UreD*[2]	Kleb 3 F3	GAT CTC CGC TTT CAG CAG
		Kleb 3 B3	CAA CTG CTG GCG AAC TAG
		Kleb 3 FIP	TCA TCA CCG CCG ACG ATG CCG TTT TAC CCG GAA GAA G
		Kleb 3 BIP	TGA CAA TTA GCG CGC ACC TTG CGG TAA AAC TTG CTG G
		Kleb 3 Floop[6]	GAA GCA GAT AGA GGT GAC AGG
		Kleb 3 Bloop[5]	CTG CCA TAC GCT GAT AAC CA
Staphylococcus aureus	*nuc*[3]	SA NUC F3-2	AAC AGT ATA TAG TGC AAC TTC AA
		SA NUC B3-2	CTT TGT CAA ACT CGA CTT CAA
		SA NUC FIP-2[5]	TGT CAT TGG TTG ACC TTT GTA CAT TAA AAT TAC ATA AAG AAC CTG CGA
		SA NUC BIP-2[6]	GTT GAT ACA CCT GAA ACA AAG CAT CAT TTT TTT CGT AAA TGC ACT TGC
		SA NUC Floop	AAC MTA TAC CAT CAA TCG CTT TA
		SA NUC Bloop	AAG GTG AGA GAA ATA TGG TCC TGA
Streptococcus spp.	*16S rRNA*[4]	Strep 3 F3	CGC AAC CCC TAT TGT TAG TT
		Strep 3 B3	GCG ATT CCG ACT TCA TGT
		Strep 3 FIP[5]	ACG TCA TCC CCA CCT TCC TCA TCA TTA AGT TGG GCA CTC TA
		Strep 4 BIP[6]	TGG TTG TTA CAA CGA GTC GCA ATC CGA ACT GAG ATT GTC
		Strep 3 Floop	TTA TTA CCG GCA GTC TCG C
		Strep 3 Bloop	TGA CGG CAA GCT AAT CTC TTA A

ECOLI=*E. coli*, Kleb=*K. pneumoniae*, SA=*S. aureus*, Strep=*Streptococcus* spp., FIP=forward inner primer, BIP=backward inner primer, Floop=forward loop primer, Bloop=backward loop primer
Assay designed using target gene as described by Shome et al. (2011)
[1] Assay designed using target gene as described by Zamani et al. (2013)
[2] Assay designed using target gene as described by Tie et al. (2012)
[3] Assay designed using target gene as described by Wang and Li (2015)
[4] Primer labeled with FAM when used in nucleic acid lateral flow immunoassay
[5] Primer labeled with biotin when used in nucleic acid lateral flow immunoassay

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Bacterial isolates and milk samples

To determine the limit of detection for each LAMP assay, four positive controls were selected and prepared as described (Cornelissen et al., 2016). The positive controls were *E. coli* strain 13-L24, *K. pneumoniae* strain 2.35*, S. aureus* strain 2.24*,* and *S. uberis* strain 2.28. Cultures of these isolates were serial diluted 5-fold in milk, starting with a 1:5 dilution. The *E. coli* suspension started with a counting of $8.0*10^7$ (standard deviation (SD) $2.8*10^7$) cfu/mL, *K. pneumoniae* with a counting of $5.0*10^8$ (SD $8.5*10^7$) cfu/mL, *S. aureus* with a counting of $1.1*10^8$ (SD $1.4*10^7$) cfu/mL, and *Streptococcus* spp. with a counting of $3.9 *10^8$ (SD $1.4*10^7$) cfu/mL. The number of cfu per mL was determined by serial dilution and plating in duplicate on sheep blood agar heart infusion plates (Wageningen Bioveterinary Research, Lelystad, the Netherlands). The milk originated from healthy cows and was checked to be culture negative for the four target pathogens.

Second, well-characterized isolates from different bacterial species (Table 2) were used to determine the analytical specificity of the LAMP assays. These well-characterized isolates from an in-house collection of well-characterized isolates (Cornelissen et al., 2016), largely originate from cows with mastitis and were supplemented with isolates from relevant species. The well-characterized isolates were cultured and harvested as described (Cornelissen et al., 2016).

The 1st set of samples, used to determine the diagnostic specificity, was a subset of the milk samples used in the study described by Griffioen et al. (2018). In that study, 866 milk samples were analyzed at the bacteriological laboratory of Royal GD (Deventer, the Netherlands) by the reference method between April and July 2016. Subsets were compiled with culture positive and negative samples for each target of the four LAMP assays (Table 2).

The 2nd set of samples were tested blindly in all four LAMP assays to determine the diagnostic performance. The set comprised 163 milk samples from cows with mild and moderate clinical mastitis collected in a field study. These field samples were collected on 15 dairy farms in the Netherlands between May 2017 and July 2018 as described in *Chapter 6*. These samples were also analyzed at the bacteriological laboratory of GD Animal Health by the reference method (Table 2).

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Table 2. Bacteriological culture combined with mass spectrometry used as reference method to determine analytical and diagnostic characteristics of four LAMP assays using well-characterized bacterial isolates from in-house collection (*n* = 128), not blindly tested selected samples (*E. coli n* = 96; *K. pneumoniae n* = 84; *S. aureus n* = 112; *Streptococcus* spp. *n* = 96) originated from cows suspected to have mastitis, and blindly tested field samples (*n* = 163) originated from cows with clinical mastitis

				Milk samples					
		Isolates		Selected samples				Blindly tested samples	
Group	**Species**	*n*	**Proportion of total**	***E. coli* LAMP assay**	***K. pneumonia* LAMP assay**	***S. aureus* LAMP assay**	***Streptococcus* spp.LAMP assay**	*n*	**Proportion of total**
No growth		0	0.00	-	-	-	-	29	0.18
Gram-positive	*Staphylococcus* spp.	30	0.23	15	16	44	24	37	0.23
	S. aureus	3	0.02	8	8	40	12	17	0.10
	Non-aureus staphylococci	27	0.21	7	8	24	12	20	0.12
	Streptococcus spp.	30	0.23	9	8	24	53	40	0.25
	S. agalactiae	7	0.05	-	-	-	-	0	0.00
	S. castoreus	2	0.02	-	-	-	-	0	0.00
	S. dysgalactiae	14	0.11	2	-	8	24	20	0.12
	S. uberis	7	0.05	7	8	16	27	19	0.12
	Other Gram-positives	18	0.14	-	-	-	-	20	0.12
Gram-negative	*Escherichia coli*	7	0.05	48	16	-	-	31	0.19
	Klebsiella spp.	11	0.09	-	32	6	-	1	0.01
	K. oxytoca	6	0.05	-	-	-	-	-	-
	K. pneumoniae	5	0.04	12	30	4	-	-	-
	Other *Enterobacteriaceae*	21	0.16	-	-	-	-	4	0.02
	Other Gram-negative	10	0.08	-	-	-	-	2	0.01
Other	*Leptospira hardjo*	1	0.01	-	-	-	-	-	-
	Other[1]	-	-	12	12	18	19	0	0.00
Contaminated[2]		0	0.00	-	-	-	-	2	0.01

[1] Other mastitis-causing pathogens than *S. aureus*, non-aureus staphylococci, *S. dysgalactiae*, *S. uberis*, hemolytic streptococci, *E. coli*, *K. pneumoniae*, or *Klebsiella* spp.
[2] More than two different species were cultured from the milk sample

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Reference method

Bacteriological culture with identification using mass spectrometry, as performed at the routine bacteriology laboratory of Royal GD, was used as reference method (Griffioen et al., 2018). In short, 10 µL of milk was inoculated onto 6% sheep blood agar plates to determine the bacteriological status. Plates were incubated aerobically for 48 h at 37°C. Growth of presumptive mastitis-causing bacteria was examined after 24 h and 48 h, according to the guidelines of the National Mastitis Council (NMC, 1999b). Presumptive mastitis-causing bacteria were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI Biotyper 3.1, Bruker Daltonics GmbH, Bremen, Germany) (Barreiro et al., 2010).

Isolation of DNA

Aliquots of 250 µL of suspended bacteria from the selected well-characterized isolates, or 500 µL of milk samples, were used to isolate template DNA, essentially as described (Cornelissen et al., 2016). The bacterial suspensions and milk samples were incubated for 1 h at 37°C with 80 µL of Tris-EDTA buffer (20 mmol/L Tris-HCl (pH 8.0), and 2 mmol/L EDTA), containing achromopeptidase (1000 U/mL), lysostaphin (20 µL/mL), lysozyme 1 (mg/mL), and mutanolysin 100 (U/mL) (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). Thereafter, 40 µL proteinase, 360 µL ATL buffer from the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands), and 400 µL AL buffer from the same kit was added, and the mix was incubated for 1 h at 56 °C. Of a mixture of 400 µL ethanol and 3 µL HCl (25%), 400 µL was added to the mix. Membranes were washed according to the guidelines of the manufacturer and the isolated DNA was eluted with 50 µL water.

LAMP reactions

For each LAMP assay, positive controls were used in the concentration of 1 ng/µL. For the analytical performance, 2 µL of the positive controls was used, for the diagnostic performance, 8 µL was used.

Loop-mediated isothermal amplification reactions were in essence performed as described (Cornelissen et al., 2016), using a commercially available mix with a polymerase with strand displacement activity and EvaGreen (Isothermal Master Mix; OptiGene, Horsham, UK). For *E. coli*, *K. pneumoniae,* and *S. aureus* the LAMP reactions were performed in a final volume of 23.0 µL, containing the isothermal mastermix, 2.0 µmol/L of each of the forward inner primer (FIP) and the backward inner primer

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(BIP), 0.2 µmol/L of each of the outer primers F3 and B3, 1.0 µmol/L of each of the backward loop and forward loop primer, template DNA (2 µL if isolates were used or 8 µL eluate if milk samples were used) and 6 µL of ROX Passive reference (Eurogentec, Liege, Belgium). For *Streptococcus* spp., the LAMP reactions were performed in a final volume of 18.5 µL, containing the isothermal mastermix, 0.8 µmol/L of each of the FIP and the BIP, 0.2 µmol/L of each of F3 and B3 primers, 0.4 µmol/L of each of the backward loop and forward loop primer, template DNA (2 µL if isolates were used or 8 µL of template DNA if milk samples were used), and 6 µL of ROX Passive reference. The LAMP assays were performed in 96-well plates and always included negative controls (water) and positive controls. Reactions were incubated at 62°C in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) for 90 cycles of 45 seconds, and thus in total for 67.5 min, which is referred to as the real-time readout. The LAMP products were detected by screening for fluorescence by EvaGreen, which is referred to as real-time readout. The threshold was manually set at 50% in the linear phase of the amplification plot of the positive control, similar to the selection of a threshold for real-time PCR (Caraguel et al., 2011). The time point at which fluorescent signals passed this threshold is referred to as time-to-positivity (Tp) and is expressed in min. To stop the reaction, the temperature was increased to 95°C to inactivate the polymerase. To confirm the identity of amplification products, melting curve analyses were performed after amplification by decreasing the temperature from 95°C to 60°C during which fluorescence was measured. Finally, to establish if actual amplification had taken place, for each sample the presence of a sigmoidal curve was checked.

LAMP using NALFIA

To determine whether LAMP has potential to be used on-site, a lateral-flow based readout that does not require equipment, i.e. NALFIA, was evaluated. To enable readout by NALFIA, FAM and biotin labeled amplicons were generated in the LAMP reactions. For that, in each primer set, two unlabeled primers were replaced with respectively a FAM and biotin labeled version (Table 2). From a practical point of view, a generic assay time was chosen for all LAMP assay to stop the reactions in the real-time PCR machine by increasing the temperature to 95°C, which was 40 min, based on the Tp with the highest total area under the receiver operator curve (AUC) for all assays. The reaction mixtures of the experiment were taken up in reaction tubes that were inserted in contamination-free cassettes (U-Star Disposable Nucleic Acid Lateral Flow Detection Units (TwistDX Limited, Maidenhead, UK). These cassettes enable analysis of labeled amplification products by NALFIA. The cassettes contain a needle to perforate a

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reaction tube upon insertion, and a reservoir in which amplification products mix with streptavidin-conjugated colloidal gold. This results in labeling of biotinylated amplicons from the LAMP reaction with red gold particles. The resulting mixture flows into the connected nitrocellulose strip along immobilized anti-FAM antibodies, where, upon interaction with red gold-amplicon-FAM complexes, amplicons are visualized without the need for special equipment.

Analytical and diagnostic performance of LAMP assays

Limit of detection

To determine the limit of detection of the LAMP assays, each spiked milk sample from the 5-fold dilution series was tested in three independent experiments. The limit of detection was calculated as the lowest mean concentration of bacteria where all three experiments gave a signal within the length of the experiment plus 3 times SD.

Analytical specificity

To determine the analytical specificity, the cut-off for the Tp was defined as the average time it took for signals to appear when testing non-target isolates, minus 3 times SD, using only data on signals that appeared within the length of the experiment. Isolates with a Tp lower than the calculated Tp cut-off were considered positive.

Table 3. Lowest concentration detected, standard deviation (SD), and limit of detection (LOD; lowest concentration + 3*SD)) in cfu/mL of four designed loop-mediated isothermal amplification (LAMP) assays using 5-fold dilutions of target bacteria spiked in milk in two types of readout: real-time readout of fluorescence in a PCR machine tested in three independent experiments and nucleic acid lateral flow immunoassays (NALFIA) tested in one experiment

		Real-time readout			**NALFIA readout**		
	Gene	**Lowest concentration[a]**	**SD**	**LOD**	**Lowest concentration**	**SD**	**LOD**
Escherichia coli	*PhoA*	$2.6*10^4$	$9.1*10^3$	$5.3*10^4$	$1.3*10^5$	$4.5*10^4$	$2.6*10^5$
Klebsiella pneumonia	*UreD*	$1.3*10^3$	$2.2*10^2$	$1.9*10^3$	$1.3*10^3$	$2.2*10^2$	$1.9*10^3$
Staphylococcus aureus	*nuc*	$1.4*10^3$	$1.8*10^2$	$2.0*10^3$	$1.4*10^3$	$1.8*10^2$	$2.0*10^3$
Streptococcus spp.	*16S rRNA*	$1.0*10^3$	$3.6*10^1$	$1.1*10^3$	$1.0*10^3$	$3.6*10^1$	$1.1*10^3$

cfu = colony forming units

[a] Tested in two independent experiments for the *Streptococcus* spp. assay

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Table 4. Sensitivity (Se), specificity (Sp), and agreement of four designed loop-mediated isothermal amplification (LAMP) assays at different steps of the evaluation process in two types of readout (fluorescence in a real-time PCR machine (RT), with sample specific runtime or NALFIA, amplicon-antibody complexes in a nucleic acid lateral flow immunoassay, with a practical generic runtime) using well-characterized bacterial isolates of an in-house collection (n = 128), selected samples being culture positive and negative (*E. coli* n = 96; *K. pneumoniae* n = 84; *S. aureus* n = 112; *Streptococcus* spp. n = 96) that originated from cows suspected to have mastitis, and field samples (n = 163)[2] that originated from cows with clinical mastitis with bacteriological culture combined with mass spectrometry as the reference method

			Compared to reference							
LAMP assay	**Sample type and readout**	**Runtime**	**TP**	**TN**	**FP**	**FN**	**Se (95% CI)**	**Sp (95% CI)**	**Cohen's kappa (95% CI)**	**PABAK**
Escherichia coli	Bacterial isolates[1] (RT)	38 min	7	120	1	0	1.00 (0.59-1.00)	0.99 (0.95-1.00)	0.93 (0.79-1.00)	
	Selected samples[2] (RT)	31 min	40	44	4	8	0.83 (0.70-0.93)	0.92 (0.80-0.98)	0.75 (0.62-0.88)	
	Selected samples (NALFIA)	40 min	45	42	6	3	0.94 (0.83-0.99)	0.88 (0.75-0.95)	0.81 (0.70-0.93)	
	Field samples[2] (RT)	31 min	17	130	2	14	0.55 (0.36-0.73)	0.98 (0.95-1.00)	0.63 (0.46-0.79)	0.80
Klebsiella pneumoniae	Bacterial isolates (RT)	25 min	5	118	5	0	1.00 (0.48-1.00)	0.96 (0.91-0.99)	0.65 (0.37-0.93)	
	Selected samples (RT)	13 min	22	52	2	8	0.73 (0.54-0.88)	0.96 (0.87-1.00)	0.73 (0.57-0.88)	
	Selected samples (NALFIA)	40 min	32	26	26	0	1.00 (0.89-1.00)	0.50 (0.87-1.00)	0.43 (0.28-0.58)	
	Field samples (RT)	13 min	1	160	2	0	1.00 (0.03-1.00)	0.99 (0.96-1.00)	0.50 (-0.10-1.00)	0.98
Staphylococcus aureus	Bacterial isolates (RT)	18 min	3	125	0	0	1.00 (0.29-1.00)	1.00 (0.97-1.00)	1.00	
	Selected samples (RT)	26 min	33	68	4	7	0.83 (0.67-0.93)	0.94 (0.86-0.98)	0.78 (0.66-0.90)	
	Selected samples (NALFIA)	40 min	36	70	2	4	0.90 (0.76-0.97)	0.97 (0.90-1.00)	0.88 (0.79-0.97)	
	Field samples (RT)	26 min	12	146	0	5	0.71 (0.44-0.87)	1.00 (0.98-1.00)	0.81 (0.65-0.97)	0.94
Streptococcus spp.	Bacterial isolates (RT)	15 min	30	98	0	0	1.00 (0.88-1.00)	1.00 (0.96-1.00)	1.00	
	Selected samples (RT)	48 min	52	36	7	1	0.98 (0.90-1.00)	0.84 (0.69-0.93)	0.83 (0.72-0.94)	
	Selected samples (NALFIA)	40 min	50	38	5	3	0.94 (0.84-0.99)	0.88 (0.75-0.96)	0.83 (0.72-0.94)	
	Field samples (RT)	48 min	38	83	39	3	0.93 (0.80-0.98)	0.68 (0.59-0.76)	0.47 (0.35-0.59)	0.48

TP = true positive, TN = true negative, FP = false positive, FN = false negative, PABAK = prevalence and bias adjusted kappa. The guideline of Landis and Koch (1977) was used to determine the magnitude of agreement (Cohen's kappa and PABAK). A value of > 0.80 was considered to be almost perfect, > 0.60-0.80 substantial, > 0.40-0.60 moderate, > 0.20-0.40 fair, > 0.00-0.20 slight, and ≤ 0.00 poor agreement.

[1] Tp cut-off was defined as the average time it took for signals to appear when testing non-target isolates, minus 3 times SD, using only data on signals that appeared within the length of the experiment.

[2] The area under the receiver operator curve was established using binary results of all samples with or without a valid signal. A valid signal was defined as a putative positive sample (i.e. a sample with a signal) with the melting temperature (Tm) of the product within the Tm range, calculated as the mean Tm of the positive controls plus or minus a margin of 2°C, and with a sigmoidal amplification curve.

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Diagnostic performance of LAMP using selected milk samples

To determine the diagnostic performance of LAMP in selected, characterized milk samples, Tp cut-offs for each LAMP assay were determined for each minute during the running time of the experiment using receiver operator curve (ROC) analyses in Stata Statistical Software (StataCorp. 2017. Release 15. College Station, TX: StataCorp LLC.). The AUC was established using binary results of all samples, i.e. positive or negative at the tested time points, based on the presence or absence of a valid signal. Accompanying 95% CI were determined around the AUC. A valid signal was defined as a putative positive sample (i.e. a sample with a signal) with the melting temperature (Tm) of the product within the Tm range, calculated as the mean Tm of the positive controls plus or minus a margin of 2 ^{o}C, and with a sigmoidal amplification curve. Samples with aberrant Tm or amplification curves were deemed negative and ignored for Tp cut-off calculations, but the results were used for calculating assay sensitivity (Se) and specificity (Sp). The Tp resulting in the highest AUC was selected as Tp cut-off. Further, to calculate the test Se and Sp, samples were designated positive or negative according to the determined Tp cut-off, and samples with an aberrant Tm or sigmoidal curve were included as negative results.

Diagnostic performance of LAMP using clinical mastitis milk field samples

The diagnostic performance of the LAMP assays using field samples was determined using the 163 clinical mastitis samples. Each result was judged positive or negative based on the three criteria, using the Tp cut-off as determined with ROC analysis over the selected samples, the Tm, and whether a sigmoidal curve was observed.

Statistical analysis

Results of each LAMP assay and both types of readout were compared with results of the reference method to calculate the diagnostic test characteristics Se, defined as the proportion of culture-positive samples with a positive LAMP result, Sp, defined as the proportion of culture-negative samples with a negative LAMP result (i.e. no signal below the Tp threshold, an aberrant Tm, or an aberrant amplification curve), with the accompanying binomial exact 95% CI at all steps of the evaluation (Dohoo et al., 2009). Furthermore, the proportion of agreement between the reference method and LAMP assays corrected for chance was calculated, known as Cohen's kappa (κ). For the field samples also the prevalence and bias corrected kappa (PABAK) was calculated (Byrt et al., 1993). The guideline of Landis and Koch (1977) was used to determine the magnitude of agreement. A value of > 0.80 was considered to be almost perfect, >

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0.60-0.80 substantial, > 0.40-0.60 moderate, > 0.20-0.40 fair, > 0.00-0.20 slight, and ≤ 0.00 poor agreement.

Results

Limit of detection

The limit of detection for both readouts were equal for all LAMP assays, except for the *E. coli* LAMP assay where the limit of detection of the real-time readout was lower (Table 3). The *Streptococcus* spp. LAMP assay was the most sensitive assay with $1.1*10^3$ cfu/mL as detection limit for both readouts.

Analytical specificity using well-characterized isolates

The analytical specificity determination showed that all LAMP assays had high Se and Sp (≥ 0.96). Also, kappa was substantial to almost perfect for the four assays to detect their target bacteria (Table 4). At this step of the evaluation, the calculated Tp cut-off for the *E. coli* LAMP assay was 38 min. The observed Tp values for *E. coli* isolates ranged from 14 to 24 min (mean 17 min). The isolate for which a false positive result was found, was an *Enterococcus durans*, with a Tp of 36 min. For the *K. pneumoniae* assay, the calculated Tp cut-off was 25 min. The observed Tp values for *K. pneumoniae* isolates ranged from 7 to 9 min (mean 8 min). The isolates for which false positive results were found, were one *E. durans* and four *K. oxytoca* isolates, which had a Tp between 17 and 24 min. For the *S. aureus* assay, the calculated Tp cut-off was 18 min. The observed Tp for *S. aureus* isolates ranged from 6 to 7 min. For the *Streptococcus* spp. assay, the calculated Tp cut-off was 15 min. The observed Tp for *Streptococcus* spp. isolates ranged from 9 to 11 min (mean 10 min). The latter two assays had only true positive and true negative results.

Diagnostic performance of LAMP using selected milk samples

The Tp cut-offs for each LAMP assay as obtained with the ROC analysis are shown in Table 5. If multiple time points were found to have the highest AUC, the point with the lowest Tp value was chosen. Applying the optimal Tp cut-off to the whole set of selected samples, resulted in Se ranging from 0.73 to 0.98, and Sp ranging from 0.84 to 0.96 for all LAMP assays (Table 6).

At the optimal Tp cut-off of 31 min for the *E. coli* assay, Se was 0.83 (Table 6). The Tp of true positive samples ranged from 15 to 31 min (mean 22 min). The average Tm of the

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positive controls was 89.0°C. Of the samples with false negative results, six samples did not give a signal within the length of the experiment or above the optimal Tp cut-off, and two had a Tm below the Tm range (average Tm 86.5°C). The four samples with false positive results were *K. pneumoniae*, *S. aureus*, *S. uberis*, and an unspecified bacterium (growth, but no *S. aureus*, *K. pneumoniae*, *E. coli*, non-aureus staphylococci (NAS), or *Streptococcus* spp.) according to the reference method.

At the optimal Tp cut-off of 13 min for the *K. pneumonia* assay, Se was 0.73 (Table 6). The Tp of the samples with true positive results ranged from 7 to 12 min (mean 8 min). The average Tm was 90.9°C. Seven of the samples that were determined to have false negative results had no signal within the length of the experiment or the Tp was too high, one had Tm of 88.8°C. The two samples with false positive results were *E. coli*, and NAS according to the reference method.

At the optimal Tp cut-off of 26 min for the *S. aureus* LAMP assay, Se was 0.83 (Table 6). The Tp of the samples with positive results ranged from 6 to 25 min (mean 11 min). The average Tm of the positive controls was 81.1°C. The samples with false negative results were scored negative based on the Tp being too high (n = 6), and one had a Tm of 79.0°C. The four samples with false positive results were *S. dysgalactiae*, NAS (n = 2), and an unspecified bacterium.

At the optimal Tp cut-off of 48 min for the *Streptococcus* spp. LAMP assay, Se was 0.98 (Table 6). The Tp ranged from 7 to 48 min (mean 29 min). The average Tm of the true positives was 87.6°C. The sample with the false negative result scored negative because no signal was detected within the length of the experiment. Of the samples with false positive results one was positive for NAS, and six contained unspecified bacteria according to the reference method.

Diagnostic performance of LAMP using clinical mastitis milk samples

The diagnostic performance of the four LAMP assays using 163 blindly tested field samples is shown in Table 4. The Se for all assays ranged from 0.55 to 1.00, and Sp from 0.68 to 1.00. Kappa ranged from 0.47 to 0.81, but was often affected by prevalence and bias as shown by PABAK.

For the *E. coli* assay the observed Tp of the samples with true positive results ranged from 17 to 30 min (mean 23 min). The samples deemed false positive by LAMP were

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S. uberis according to the reference method. Of the positive samples according to the reference method, 14 were deemed negative by LAMP based on the Tp being too high or not detected (*n* = 13), or because the Tm was outside of the Tm range of 86.3 to 90.3°C (*n* = 1; 75.3°C).

Table 5. Area under the receiver operator curve (AUC) of four LAMP assays at different runtimes at which it was assessed whether a sample had a signal or not, to determine the optimal Tp cut-off (shown in bold). Selected positive and negative milk samples according to bacteriological culture with identification by mass spectrometry for each target were used that had a signal within 67.5 min, but without an aberrant melting temperature (based on the melting temperature of the positive controls) or sigmoidal curve

	***Escherichia coli* (*n* = 90)**		***Klebsiella pneumoniae* (*n* = 78)**		***Staphylococcus aureus* (*n* = 103)**		***Streptococcus* spp. (*n* = 89)**	
Runtime (min)	**AUC**	**95% CI**	**AUC**	**95% CI**	**AUC**	**95% CI**	**AUC**	**95% CI**
10	-	-	0.79	0.70-0.88	0.69	0.61-0.77	0.52	0.49-0.54
11	-	-	0.83	0.74-0.92	0.80	0.72-0.88	0.52	0.49-0.54
12	-	-	0.84	0.76-0.93	0.85	0.78-0.93	0.52	0.49-0.54
13	-	-	**0.87**	**0.79-0.95**	0.86	0.78-0.93	0.52	0.49-0.54
14	-	-	0.86	0.77-0.94	0.87	0.80-0.94	0.52	0.49-0.54
15	0.51	0.49-0.53	0.85	0.76-0.93	0.88	0.81-0.95	0.52	0.49-0.54
20	0.66	0.59-0.73	0.78	0.69-0.87	0.89	0.82-0.96	0.54	0.50-0.57
25	0.78	0.70-0.86	0.77	0.68-0.85	0.90	0.84-0.97	0.58	0.51-0.66
26	0.80	0.73-0.88	0.77	0.68-0.85	**0.92**	**0.86-0.97**	0.58	0.51-0.66
27	0.84	0.76-0.91	0.77	0.68-0.86	0.92	0.86-0.97	0.60	0.52-0.68
28	0.87	0.80-0.94	0.76	0.66-0.85	0.92	0.86-0.97	0.66	0.58-0.74
29	0.88	0.81-0.95	0.75	0.65-0.84	0.92	0.86-0.97	0.69	0.61-0.78
30	0.88	0.81-0.95	0.75	0.65-0.84	0.92	0.86-0.97	0.73	0.65-0.81
31	**0.89**	**0.82-0.95**	0.75	0.65-0.84	0.92	0.86-0.97	0.76	0.68-0.84
32	0.89	0.82-0.95	0.73	0.63-0.82	0.92	0.86-0.97	0.80	0.72-0.88
33	0.89	0.82-0.95	0.71	0.61-0.80	0.92	0.86-0.97	0.80	0.72-0.88
34	0.89	0.82-0.95	0.71	0.61-0.80	0.92	0.86-0.97	0.81	0.73-0.89
35	0.89	0.82-0.95	0.71	0.61-0.80	0.92	0.86-0.97	0.82	0.74-0.90
40	0.89	0.82-0.95	0.71	0.61-0.80	0.92	0.86-0.97	0.87	0.80-0.95
45	0.89	0.82-0.95	0.71	0.61-0.80	0.90	0.84-0.96	0.88	0.81-0.93
46	0.89	0.82-0.95	0.71	0.61-0.80	0.89	0.83-0.96	0.88	0.81-0.94
47	0.89	0.82-0.95	0.71	0.61-0.80	0.91	0.85-0.96	0.88	0.81-0.95
48	0.89	0.82-0.95	0.71	0.61-0.80	0.90	0.84-0.96	**0.89**	**0.83-0.96**
49	0.89	0.82-0.95	0.71	0.61-0.80	0.89	0.83-0.95	0.87	0.79-0.94
50	0.89	0.82-0.95	0.71	0.61-0.80	0.89	0.83-0.95	0.87	0.79-0.94

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Only one field sample was positive for *K. pneumoniae* according to the reference method, for which also the *K. pneumoniae* LAMP assay was positive. Two sample were deemed false positive by LAMP, one from which *Bacillus pumilus* was recovered according to the reference method, and for the other no growth was reported.

Table 6. Sensitivity (Se), Specificity (Sp) of four LAMP assays at different runtimes. To calculate Se and Sp for each possible runtime (min), for selected milk samples the results of LAMP assays (absence or presence of a valid signal below the optimal Tp cutoff) were compared to results of the reference method (bacteriological culture with identification by mass spectrometry). The Se and Sp at the optimal Tp are shown in bold

	***Escherichia coli* (*n* = 96)**		***Klebsiella pneumoniae* (*n* = 84)**		***Staphylococcus aureus* (*n* = 112)**		***Streptococcus* spp. (*n* = 96)**	
Runtime (min)	**Se**	**Sp**	**Se**	**Sp**	**Se**	**Sp**	**Se**	**Sp**
10	-	-	0.57	1.00	0.35	1.00	0.04	1.00
11	-	-	0.63	1.00	0.55	1.00	0.04	1.00
12	-	-	0.67	1.00	0.65	1.00	0.04	1.00
13	-	-	**0.73**	**0.98**	0.68	0.99	0.04	1.00
14	-	-	0.77	0.93	0.70	0.99	0.04	1.00
15	0.02	1.00	0.77	0.91	0.75	0.96	0.04	1.00
20	0.31	1.00	0.80	0.76	0.78	0.94	0.08	1.00
25	0.58	0.96	0.83	0.70	0.80	0.94	0.25	0.93
26	0.63	0.96	0.83	0.70	**0.83**	**0.94**	0.25	0.93
27	0.69	0.96	0.83	0.70	0.83	0.94	0.28	0.93
28	0.75	0.96	0.83	0.69	0.83	0.94	0.40	0.93
29	0.79	0.94	0.83	0.67	0.83	0.94	0.47	0.93
30	0.79	0.94	0.83	0.67	0.83	0.94	0.55	0.93
31	**0.83**	**0.92**	0.83	0.67	0.83	0.94	0.60	0.93
32	0.83	0.92	0.83	0.63	0.83	0.94	0.68	0.93
33	0.83	0.92	0.83	0.59	0.83	0.94	0.72	0.91
34	0.85	0.90	0.83	0.59	0.83	0.94	0.75	0.88
35	0.85	0.90	0.83	0.59	0.83	0.94	0.77	0.88
40	0.85	0.90	0.83	0.59	0.83	0.94	0.94	0.84
45	0.85	0.90	0.83	0.59	0.83	0.92	0.96	0.84
46	0.85	0.90	0.83	0.59	0.83	0.90	0.96	0.84
47	0.85	0.90	0.83	0.59	0.85	0.90	0.96	0.84
48	0.85	0.90	0.83	0.59	0.85	0.89	**0.98**	**0.84**
49	0.85	0.90	0.83	0.59	0.85	0.88	0.98	0.79
50	0.85	0.90	0.83	0.59	0.85	0.88	0.98	0.79
60	0.85	0.88	0.83	0.56	0.88	0.82	0.98	0.77
70	0.85	0.83	0.87	0.56	0.88	0.79	0.98	0.74

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For the *S. aureus* assay the observed Tp of the samples with true positive results ranged from 7 to 15 min (mean 11 min). None of the samples was deemed false positive by LAMP. Of the positive samples according to the reference method, five were deemed negative by LAMP because no signal was found.

For the *Streptococcus* spp. assay, the observed Tp of the samples with true positive results ranged from 6 to 47 min (mean 15 min). The Tp of the samples with false positive results ranged from 4 to 47 min (mean 23 min). From the samples with false positive results in the *Streptococcus* spp. assay, the reference method recovered no bacteria (n = 9), NAS (n = 9), *E. coli* (n = 5), *S. aureus* (n = 4), *Corynebacterium* spp. (n = 2), and the following pathogens were all once detected: *Enterococcus saccharolyticus*, *K. pneumoniae*, both NAS and *Enterococcus faecalis*, *S. aureus* and *E. coli*, *E. coli* and Gram-positive cocci, *Lactococcus garvieae*, contamination, *Serratia* spp., *Candida*, or *Enterococcus faecium*. The *Streptococcus* spp. positive samples according to the reference method deemed negative by LAMP had a Tp above the cut-off (n = 1), or no signal (n = 2).

4

NALFIA readout

Results of the NALFIA readout using the selected milk samples are shown in Table 4. A practical generic runtime of 40 min was selected, based on the highest AUC at that runtime over all assays using selected characterized samples in the diagnostic performance analysis. The Se ranged from 0.90 to 1.00 over all LAMP assays, and the Sp ranged from 0.50 to 0.97. For the samples with false positive results in the *E. coli* assay, the reference method resulted in *K. pneumoniae* (n = 5) and *S. aureus* (n = 1). For the samples with false positive results in the *K. pneumoniae* assay, the reference method resulted in *E. coli* (n = 13), followed by *S. aureus* (n = 5), NAS (n = 4), *S. uberis* (n = 1), and unspecified bacteria (n = 3). For the samples with false positive results in the *S. aureus* assay, the reference method resulted in *S. dysgalactiae*, and an unspecified bacterium. For the samples with false positive results in the *Streptococcus* spp. assay, the reference method resulted in NAS (n = 2), *S. aureus* (n = 1), and unspecified bacteria (n = 2).

Discussion

Fast, sensitive, and on-site tests are preferred by farmers to target their treatments in case of clinical mastitis (Griffioen et al., 2016). This study aimed to develop and to

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evaluate such assays. The four developed LAMP assays had high Se, Sp (both ≥ 0.73), and kappa (≥ 0.65) compared to the reference method to detect *E. coli*, *K. pneumonia, S. aureus*, and *Streptococcus* spp., both for the well-characterized isolates and for selected characterized milk samples. For field samples, the Se was lower for 3 of 4 assays, but PABAK was ≥ 0.80, except for the *Streptococcus* spp. assay. Therefore, the agreement between the reference method and the *E. coli*, *K. pneumoniae* and *S. aureus* LAMP assays was considered to be almost perfect. Most kappa estimates were influenced by the prevalence of the target bacteria in the field samples. In case of a low (or high) prevalence for the target of the assay, no high kappa values can be obtained as most of the results are deemed true positive or true negative (Byrt et al., 1993), and thus a skewed distribution of results would be obtained. Therefore, we also included PABAK in the results to correct for that effect, which is reflected most clearly for the *K. pneumoniae* assay used on the field samples, where the agreement changed from 0.50 (kappa) to 0.98 (PABAK) after correction for the low prevalence of *K. pneumoniae*.

The test performance was perfect using the well-characterized isolates as showed by the high Se and Sp estimates. However, the assays were less sensitive (higher limit of detection) than the reference method, which has a limit of detection of approximately 100 cfu/mL (Ruegg, 2018). This probably caused the low Se found for the *E. coli* LAMP assay used on the blindly tested field samples. Only one design was investigated, it is possible that in the chosen target region variation is larger than anticipated, and that another design would result in a more sensitive assay. Most further evaluation steps for the evaluated assays showed high Se and Sp estimates compared to the reference method. However, two assays had low Sp, namely the *K. pneumoniae* assay using the NALFIA as readout on the selected characterized milk samples, and the *Streptococcus* spp. assay using the fluorescence readout on the blindly tested field samples. One explanation might be that there is a discrepancy between the library of the MALDI Biotyper and the NCBI database as we found in the analytical specificity evaluation a *K. pneumoniae* positive result with the LAMP assay where this isolate was characterized as *K. oxytoca*. More likely is that the false positive results for *K. pneumoniae* in the NALFIA readout were caused by the generic assay time we had chosen for all four assays, which was longer than the optimal Tp. In total, the AUC was highest for all four assays at 40 min and thus this Tp cut-off was selected and used as a practical generic runtime for the NALFIA readout. This runtime affected the results only slightly for the *E. coli*, *S. aureus* and *Streptococcus* spp. assays. For *K. pneumoniae*, however, the runtime of 40 min increased Se, but lowered the Sp from 0.98 to 0.59, and thus

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too many samples were deemed false positive, as compared to the reference method. Clearly, a generic runtime combined with NALFIA readout is only possible when the LAMP assays have a comparable Tp cut-off. For the present assays, that would require a (new) compatible LAMP assay for *K. pneumoniae* with a Tp cutoff around 40 min. For the *Streptococcus* spp. assay, the high number of false positive results in the blindly tested field samples might be explained by differences in specificity between the reference method and the LAMP assay: the reference method does not report irrelevant growth, such as environmental streptococci that may be present in the milk, but likely are not associated with mastitis. The LAMP assay, however, was developed as genus assay instead of a species assay and thus detected all *Streptococcus* spp. This effect is seen more often when culture is compared to DNA techniques such as PCR (Koskinen et al., 2010; Taponen et al., 2010; Oikonomou et al., 2012), and could also have affected the results of *K. pneumoniae* assay as being an abundant pathogen in the environment of the cow (Zadoks et al., 2011). Collecting milk samples is sensitive to contamination, and thus environmental bacteria may be present in the milk, without being associated with mastitis. Thus, proper sample collection is important to obtain reliable test results, but might be even more important when on-site tests will be applied. Nevertheless, this type of misclassification for the *Streptococcus* spp. LAMP assay would not lead to problems from a treatment perspective in practice, because most samples with false positive results contained Gram-positive bacteria according to the reference method, and thus the treatment applied would probably also affect these Gram-positive bacteria. From the perspective of prudent antimicrobial use, however, these misclassifications are unwanted. Thus, to obtain a test that results in relevant outcomes, further research should be performed on more samples collected in the field to determine the criteria necessary to judge samples for the *K. pneumoniae* and *Streptococcus* spp. LAMP assay.

We used three criteria to judge the results of the LAMP assays: whether a signal emerged within in the length of the experiment, the presence of a sigmoidal amplification curve and the Tm of the amplification product. The Tm of amplicons is determined by length and composition of the amplicon, and in part by the amount of template DNA and the amount of dye in a reaction (Monis et al., 2005; Reed et al., 2007), and differs among different strains of the same species (Guion et al., 2008). Thus, if Tm is used as a judgment criterion, a range rather than a single Tm is demanded. Only one positive control strain was used to determine the Tm of de LAMP products. As the variation of the products in field strains is not known, an arbitrary 2°C margin around the mean was used. It is conceivable that with a larger set of strains this range can be finetuned.

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Thus, further research is necessary to better understand the effects of the different criteria used, specifically to judge the results of the *K. pneumoniae* and *Streptococcus* spp. assay on multiple bacterial species.

All LAMP reactions were performed in a real-time PCR machine. However, the ultimate goal is to develop a test that can be used on-site. For that, all steps, from sample preparation to the readout of the result, should be easy to execute, with minimal and simple equipment. As a first step towards on-site LAMP, the feasibility of NALFIA as readout was investigated; isolating DNA and performing of the assay was still done with protocols and equipment that require a laboratory. We found that the limit of detection was comparable between the real-time and NALFIA readout. From a practical perspective, we chose to work with a generic runtime of 40 min for all LAMP reactions as to make the test suitable for parallel on-site testing. When tested with the selected milk samples, the NALFIA readout had an almost perfect agreement with the reference method, except for *K. pneumoniae* with a kappa of 0.40. Most of the samples with false positive results in the *K. pneumoniae* assay contained *E. coli* according to the reference method. If, therefore, the *K. pneumonia* NALFIA assay would be used in the field, this result would not be ideal, but the type of therapy would not be affected for most cows. For both an *E. coli* and a *K. pneumoniae* infection, as being Gram-negative bacteria, the same therapy would be applied. Nevertheless, if NALFIA would be used as readout, as for every endpoint readout the reaction should be stopped at the optimal Tp cut-off to obtain optimal results. Moreover, as ideally the assay should be run in multiplex on the same sample and for practical reasons a generic runtime is preferred, new designs are necessary for the *K. pneumoniae* and *Streptococcus* spp. assay.

Three points used in the current study make the method used not yet suitable for on-site use: the DNA extraction, the equipment, and a practical approach to test all LAMP assays in parallel on DNA of the same sample. First, although LAMP in itself is a fast test, the time needed to extract DNA from milk as performed in this study was time-consuming and resulted in a total time-to-result of about 6-8 h for the LAMP assays. Nevertheless, that is faster than culture and lies within the time-to-result indicated as preferred by farmers (Griffioen et al., 2016). However, LAMP generally is described as a test that is less demanding in terms of DNA purification than real-time PCR. For example, diluted virus stocks were directly added to the reaction mix to perform the LAMP reaction or centrifuged pellet samples were boiled with Triton X-100 after which the lysates were used for the LAMP reactions (Kaneko et al., 2007; Sowmya et al., 2012).

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Therefore, simple, somewhat crude methods for sample preparation might suffice for LAMP (Cremonesi et al., 2006; Cressier and Bissonnette, 2011; Sowmya et al., 2012; Lee et al., 2019), also for mastitis milk samples. We performed limited pilots to assess the feasibility of simple sample preparation, but so far did not find an adequate method that was simple and yet retained the same analytical sensitivity. Therefore, further research is necessary to find a simplified sample preparation without deteriorating test characteristics. Second, different studies report on the possibility of a less expensive or portable machine for running the amplifications (Lee et al., 2019; Wilisiani et al., 2019) to be used on-site (Tangkanchanapasa et al., 2018). We performed preliminary experiments with a LAMP assay for *S. uberis* (data not shown) using a portable battery powered instrument designed for performing on-site LAMP (Genie II, Optigene Ltd., Horsham, West Sussex, UK), which resulted in similar results as described (Cornelissen et al., 2016). As LAMP reactions are performed at a constant temperature, even a simple heating block will be sufficient to perform the LAMP reaction (Tangkanchanapasa et al., 2018). Furthermore, additional control criteria on Tm or sigmoidal curve to judge the results are not available in an on-site readout like NALFIA. Thus, it is important to thoroughly evaluate the designed assays to know the effects in the field. Generally, a species assay may be preferred over a genus assay to avoid high numbers of false positive results, as explained for the *Streptococcus* spp. LAMP assay. Because the results we found were comparable between both readouts, ignoring additional criteria like the Tm and sigmoidal amplification curve could be considered. To assess the performance of these LAMP assays with a simple DNA extraction protocol and a heating block would be the next step to develop on-site tests for mastitis-causing pathogens.

In conclusion, LAMP is a promising method for fast on-site tests for mastitis-causing pathogens. The *E. coli*, and *S. aureus* had a kappa ≥ 0.75 with the reference method. For the *K. pneumoniae* and *Streptococcus* spp. however, the design was suboptimal. As a consequence, *K. pneumoniae* in the NALFIA readout and the *Streptococcus* spp. assay on the blindly tested field samples resulted in a high number of false positive results. Nevertheless, the NALFIA is an easy and reliable readout for on-site use as the results were similar to the results obtained using the real-time readout, with that remark that a shorter runtime needs to be performed for the *K. pneumoniae* assay. When a simplified sample preparation protocol becomes available, the combination of LAMP and NALFIA has the potential to enable fast and reliable on-site testing on milk from cows with clinical mastitis.

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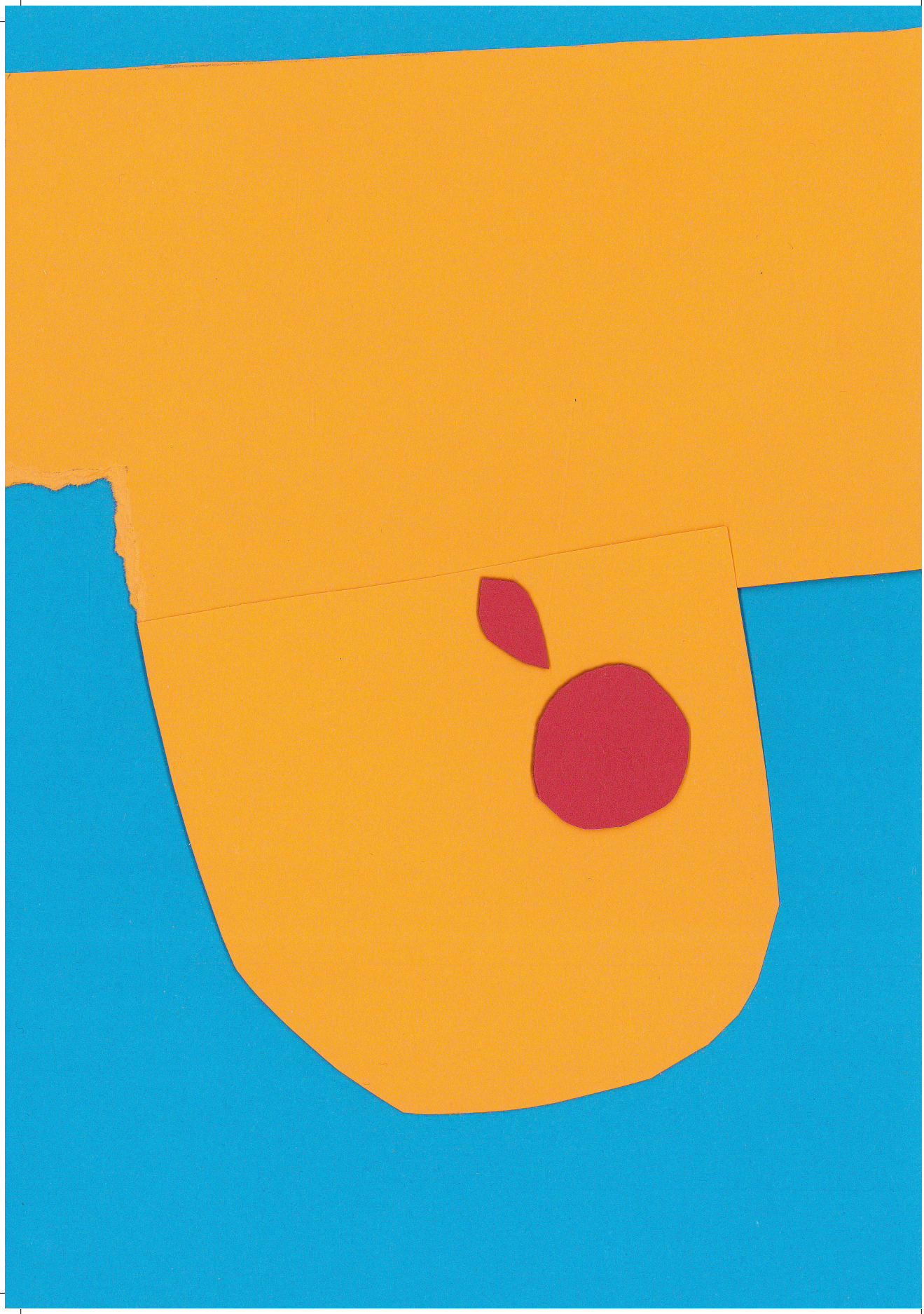

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The need for and effect of incorporating bacteriological results in dry-cow treatment decision-making

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Abstract

The study described in this research paper aimed to determine the need of dairy farmers for additional diagnostics at drying off, their interest in quarter or cow level dry-cow treatment (DCT) decision-making, and the effect of having bacteriological culture (labBC) results available on their DCT decisions. The study included two components: a survey among 292 dairy farmers to assess the need for additional diagnostic tests to substantiate DCT decisions, and a field study with nine farmers, where milk samples were collected of 73 cows prior to drying off to be cultured with labBC. The latter farmers made two quarter level DCT decisions: the first decision was made based on the information farmers usually use for DCT decisions, the second decision was made three days later when labBC results were obtained by the farmers. Both decisions were compared to labBC results, where growth ought to be treated, to determine their Se and Sp.

The farmers participating in the survey questioned their DCT decisions most often when cow somatic cell count (CSCC) was in close proximity to indicated cut-off values for antimicrobial treatment. Most farmers indicated to need an additional cheap and reliable test to use for DCT decision-making that indicates whether to apply antimicrobials. In the field study, farmers indicated to be interested to dry off at the quarter level, but hesitated to do so in this study. If labBC results were incorporated in DCT decision-making Se as well as the antimicrobial usage increased compared to the first decision. To use labBC selectively to decide on DCT for cows with CSCC ≥ 200,000 cells/mL, and leaving all cows with CSCC < 200,000 cells/mL untreated, would optimise DCT decisions and decrease antimicrobial usage as compared to the current strategy.

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Introduction

Until recently it was common practice in many countries to dry off all cows with intramammary applied antimicrobials, known as blanket dry-cow treatment (DCT). However, antimicrobial usage became subject to discussion due to its potential effect on the development of antimicrobial resistance (Levy and Marshall, 2004). Consequently, preventive use of antimicrobials in animals, including blanket DCT, is under debate, (Scherpenzeel et al., 2016). To use antimicrobials curatively at drying off, cows likely having an intramammary infection (IMI) need to be selected for DCT (Halasa et al., 2009a), as has been practiced for up to 40 years in some countries (Østerås et al., 1999; Ekman and Østerås, 2003).

Different tests are available for farmers to screen cows for IMI, like cow somatic cell count (CSCC) as recorded on test-day records from milk recording programs (Bradley and Green, 2004; Scherpenzeel et al., 2016), or the California Mastitis Test (Dingwell et al., 2003). Numerous studies describe test characteristics of available tests to find IMI, with varying results (Poutrel and Rainard, 1981; Bhutto et al., 2012; Fosgate et al., 2013; Nyman et al., 2016; Gohary and McDougall, 2018). Most of these tests diagnose an IMI indirectly, but are unable to identify the mastitis pathogen involved. Consequently, such tests do not support decisions to apply antimicrobials and cannot be used to decide on the type of antimicrobial treatment. As a result, if farmers use such diagnostic approaches, incorrect treatment decisions may be taken (Harmon, 1994; Scherpenzeel et al., 2014; Jashari et al., 2016). Tests that identify mastitis pathogens like bacteriological culture of milk provide specific information on the IMI, and thus lead to more targeted DCT decisions (Østerås and Sølverød, 2009), without affecting udder health compared to blanket DCT (Cameron et al., 2013; Cameron et al., 2014). Besides that, they provide information at the quarter level. Quarter level DCT decisions might be the next step to target DCT decisions even more, and to further reduce antimicrobial usage (Robert et al., 2006). However, whether farmers are interested in DCT decision-making at the quarter level is unknown.

In most countries sufficient possibilities for bacteriological culture in laboratories (labBC) are available. Nevertheless, most dairy farmers (85%) do not use bacteriological diagnostics at drying off, although up to 31% indicates to prefer additional information to determine whether to apply antimicrobials at drying off (Griffioen et al., 2016). Reasons

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for not using diagnostics in addition to CSCC at drying off might be, for example, the time lag between sampling and result, although that should not be an issue at drying off.

Decision-making is a dynamic process and many factors influence the final decision. Farmers not only use CSCC of test-day records to decide on DCT, they also use information such as (sub)clinical mastitis history and milk yield on the day of drying off (Rajala-Schultz et al., 2011; Scherpenzeel et al., 2016; Vasquez et al., 2018; Vilar et al., 2018). Consequently, test characteristics are not the only factor that influence decisions taken (Siontis et al., 2014). Therefore, all aspects of decision-making should be incorporated to evaluate the effect of diagnostics on the final decision.

This study aimed to determine the need of farmers for additional diagnostics at drying off, their interest in quarter or cow level DCT decision-making, and the effect of having labBC results available on DCT decisions made by farmers.

Materials and Methods

Study design

This study included two components: a telephone survey conducted between November 2016 and December 2016 among 292 randomly selected Dutch dairy farmers to assess the need of farmers for additional diagnostic tests to substantiate DCT decisions, and a field study conducted between October 2016 and February 2017 using a convenience sample of nine dairy farms. On these farms milk samples were collected of 73 cows prior to drying off and farmers made quarter level DCT decisions based on two different sources of information. They were also interviewed to assess their interest in and preferences for an additional test at drying off.

Telephone questionnaire

The 30-minute telephone questionnaire consisted of questions regarding the interest of farmers for quarter or cow level decision-making, and their need for additional diagnostic information at drying off. The questionnaire comprised open questions, questions with predefined answer options that were not read out loud, and closed questions to be scored on a Likert scale (Likert, 1932). The questionnaire is available upon request from the corresponding author. The questionnaire was conducted by four trained interviewers with a background in animal husbandry. The person responsible

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for udder health management at the farm was requested to answer the questionnaire. Not all questions were answered by all farmers due to the routing in the questionnaire. One question regarding farmers' uncertainties about DCT decisions was added after the first ten interviews were conducted.

Questions and answers of the telephone questionnaires were digitalized with NetQ (Collector 2015.Q2, Survalyzer. Utrecht, the Netherlands). If the farmer gave an answer that differed from the predefined answers, the answer was put in an open answer box and was categorized later.

Field study

Farm and cow selection

A cooperating veterinary clinic in the province of Flevoland, the Netherlands, provided contact details of nine dairy farmers. The farmers were contacted by telephone to provide study details. All farmers were willing to participate.

Cows to be dried off between October 2016 and January 2017 were eligible to participate in the study. Cows were excluded if they had clinical mastitis at the moment of sample collection or if they had received antimicrobials during the 30 days prior to sampling. No selection of cows was made based on somatic cell count (SCC).

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Sampling procedure

Per farm milk samples of eight cows were collected in two to four farm visits. The first cows that were planned to be dried off up within seven days after the farm visit were selected for that purpose. Per cow, one composite sample and a sample of each lactating quarter was collected prior to milking. Udder and teats were cleaned, and milk was collected aseptically as described earlier (NMC, 1999). All samples were collected by the same researcher and were kept refrigerated until they were cultured within 24 h.

Classical bacteriological culture

Classical labBC was conducted according to standard protocols, based on the guidelines of the NMC (1999). In short, a disposable plastic loop was used to inoculate 10 µL of milk on 6% sheep blood agar plates (bioTRADING, Mijdrecht, the Netherlands). The plates were incubated at 37 ˚C and examined after 24 ± 3 h and 48 ± 3 h. Classical labBC was considered positive if one or more colony forming units (cfu) were present, negative when no cfu were present ('no growth') and considered contaminated when three

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or more phenotypically different cfu types were found on a plate. Bacteria present were identified using matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (Biotyper 3.0 Bruker Daltonics, Bremen, Germany) (Barreiro et al., 2010). Results of labBC were categorized in four categories: Gram-positive, Gram-negative, no growth, and contamination (Table 1). Gram-positive bacteria were further categorised into staphylococci (with non-aureus staphylococci and *Staphylococcus aureus* separate), streptococci (with *Streptococcus uberis* separate), and other Gram-positive bacteria.

Table 1. Results of classical bacteriological culture of milk samples collected up to 7 days prior to drying off. Samples can be positive for two bacterial species

	Composite milk samples (*n* = 73)		**Quarter milk samples (*n* = 287)**	
Bacterial species	*n*	**%**	*n*	**%**
Culture negative	10	13.7	102	35.5
Gram-negative	0	0.0	1	0.3
Gram-positive	55	75.3	163	56.8
Staphylococcus spp.	26	35.6	51	17.8
Non-aureus staphylococci	24	32.9	46	16.0
S. aureus	3	4.1	5	1.7
Streptococcus spp.	9	12.3	19	6.6
S. uberis	4	5.5	5	1.7
Other Gram-positive	26	35.6	108	37.6
Contaminated samples[1]	8	11.0	21	7.3

[1] a sample was considered contaminated when three or more phenotypically different cfu types were found on a plate

DCT decisions made by farmers

The nine farmers were asked to make two DCT decisions for each lactating quarter of the participating cows. The first decision was made during the farm visit when milk samples were collected. For this DCT decision, farmers were asked to decide on DCT the way they currently do, and was defined as the default decision. Generally, this DCT decision is mainly based on the CSCC of the last test-day record of the national Dairy Herd Improvement program (CRV, the Netherlands) before drying off, where the national guideline on SDCT indicates an antimicrobial treatment for high CSCC cows (primiparous cows > 150,000 cells/mL and multiparous cows > 50,000 cells/mL) (Vanhoudt et al., 2018). Also, mastitis history of the current lactation, and milk yield at drying off are often considered in this decision (Scherpenzeel et al., 2016). The second DCT decision for each cow was asked three days later, when farmers obtained the labBC results. Farmers likely added this labBC information to the default available information when they made this

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second decision, which was defined as the BC decision. For both decisions it was asked why antimicrobials were applied, whether the farmer was uncertain about the decision made and if so, why that was the case. For the BC decision it was asked whether the decision changed after the labBC result was obtained and why. At the first farm visit it was additionally asked whether the farmer would be interested in an additional test indicating whether a bacterium is present, which bacterium is present, whether to apply antimicrobials, or which antimicrobial should be applied. Of each participating cow parity and CSCC from the last test-day record was collected.

Semi-structured face to face interviews

One interviewer performed the semi-structured individual face to face interviews with all persons responsible for udder health management at the nine participating farms during the last farm visit in February 2017. At three farms, this resulted in interviewing more than one person. The semi-structured interviews consisted of open questions regarding the parameters currently used for DCT decisions, their need for additional diagnostic information at drying off, and the desired characteristics of such additional diagnostic tests.

All interviews were recorded using a voice recorder after permission was given by the interviewee (all agreed). The interviews were subsequently transcribed in their full length with Microsoft Word (Microsoft Office Standard 2010). This transcript was imported in ATLAS.ti 1.6.0. (2017) to code the answers by open coding: labels were given to all statements per interview, followed by axial coding in which these labels were categorized in themes across the interviews (Corbin and Strauss, 1990).

Statistical analyses

Descriptive statistics of the telephone questionnaire and the semi-structured interview in the field study were performed using Stata (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

DCT decisions made by farmers

Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and kappa of the two farmer-made DCT decisions were determined as described by Dohoo et al. (2009). Therefore, we compared the DCT decisions to labBC results of two types of samples: quarter and composite milk samples. We defined that samples with no growth on labBC should not be treated with antimicrobials, whereas samples

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with growth on labBC ought to be treated with antimicrobials at drying off, regardless of the number of cfu, and the type of growth. Contaminated labBC results were excluded from this analysis. For the composite sample comparison, we defined that if the DCT decision was to apply antimicrobials to at least one quarter, the whole cow would be treated with antimicrobials. For kappa the guideline according to Landis and Koch (1977) was used to determine the magnitude of agreement. A value of > 0.80 was considered to be almost perfect, > 0.60-0.80 substantial, > 0.40-0.60 moderate, > 0.20-0.40 fair, > 0.00-0.20 slight, and ≤ 0.00 poor agreement.

In real life farmers might use labBC selectively for cows with certain SCC levels. For that reason, two hypothetical strategies were evaluated to determine how DCT decisions were affected by labBC results if applied selectively and how the antimicrobial usage changed compared to the default decision. The first scenario (a) was to hypothetically dry off all low SCC cows without antimicrobials and to apply the BC decision made by farmers to high SCC cows only. The second scenario (b) was to hypothetically dry off all high SCC cows with antimicrobials and to apply the BC decision made by farmers to low SCC cows only. For the two hypothetical strategies cows were classified as being a high SCC cows if CSCC ≥ 200,000 cells/mL on the last test-day record, or as a low SCC cow if CSCC < 200,000 cells/mL (Dohoo and Leslie, 1991).

Results

Characteristics of the participating herds (telephone survey and field study) and cows (field study) are shown in Table 2. In the field study, eight farmers participated with eight cows, and one with nine cows resulting in a total of 287 quarter samples, and 73 composite milk samples. From the samples, Gram-positive bacteria were cultured most often (Table 1). For 38 cows at least one quarter was culture negative, and for eight cows all lactating quarters were culture negative.

In the telephone questionnaire, 86% of the 292 farmers indicated to only dry off cows at the cow level, 10% both at the cow and quarter level, and 4% to dry off all their cows without antimicrobials. If the farmer indicated that DCT was applied both at the cow and quarter level, median 5% (mean 17%, range 1 to 95%) of the cows was dried off at the quarter level. Farmers (n = 31) indicated to dry off at the quarter level to save costs (23%), to improve the health of the cow (19%), if it is clear which quarter

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to treat (19%), to increase public health by lowering antimicrobial usage (13%), and because of increased awareness of animal health management and medicine usage (3%). These farmers also dried off at the quarter level if one quarter had clinical mastitis in the current lactation (65%) or if only one quarter had high SCC or conductivity (32%). Farmers (*n* = 255) indicated to not dry off at the quarter level, because no good data were available to decide at the quarter level (20%), they never thought of doing it differently than they currently do (18%), it is not a wise thing to do (13%), it is too time consuming (12%), it has no added value (8%), they want to be sure (6%), it is too costly (6%), it was not advised by the veterinarian (5%), the interdependence of quarters (4%), or because they had not enough knowledge to dry off at the quarter level (1%).

Table 2. Characteristics of herds and cows as compared to the Dutch average

Variable	Level	Telephone questionnaire	Field study	Dutch average
Participating herds				
Herd size (*n*; > 2y)	Mean	95	185	101[1]
	Median	85	131	-
Milking system (%)	Conventional	71	89	79[2]
	Automatic	29	11	21
Milk yield per year per cow (kg)		-	9,147	8,209[1]
Bulk tank milk SCC[3] (x1,000 cells/mL)		-	171	192[4]
Outdoor grazing (%)		-	22	79[5]
Age of farmer (y)		47	43	52[4]
Cows dried off with antimicrobials (%)		45	65	52[6]
Participating cows				
Parity	1	-	26 (36%)	-
	> 1	-	47 (64%)	-
Cow SCC[7]	Low	-	55 (75%)	-
	High	-	18 (25%)	-
Number of lactating quarters	4	-	68	-
	3	-	5	-

[1] Statistics Netherlands (2016)
[2] Stichting KOM (2019)
[3] Somatic cell count
[4] Qlip (2016)
[5] Duurzame Zuivelketen (2016)
[6] Vanhoudt et al. (2018)
[7] Low SCC < 200,000 cells/mL, high SCC ≥ 200,000 cells/mL

Of the farmers that answered the question regarding uncertainties about DCT decisions (*n* = 282), 37% expressed to never question the DCT decision whereas 63%

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indicated to question the decision more or less often for reasons shown in Table 3. The most frequently mentioned reason (44%) to question the decision to use or not use antimicrobials was a CSCC on the last test-day record before drying off being in close proximity of the cut-off point. When uncertain about their DCT decision, 69% of the farmers generally decided to use antimicrobials, 25% to generally not use antimicrobials, and 6% of the farmers had no predominant choice whether to use or not to use antimicrobials.

Table 3. Reasons for farmers to question their dry-cow treatment decision

	Farmers (*n* = 282)	
Indicated reason[1]	*n*	**%**
Never questioned	104	36.9
CSCC of last test-day record in close proximity of cut-off point for antimicrobial usage	74	26.2
High milk production at drying off	28	9.9
High CSCC on test-day records in current lactation and low last CSCC	25	8.9
No clinical mastitis in current lactation and high CSCC in last test-day record	21	7.4
Clinical mastitis in current lactation and low CSCC in last test-day record	19	6.7
Other reasons	17	6.0
Clinical mastitis in current lactation	14	5.0
Time to calving shorter than withdrawal period	9	3.2
Hot weather	5	1.8
Clinical signs of mastitis	5	1.8
Low production at drying off	5	1.8
Milk leakage in cubicles	5	1.8
High electronic conductivity at drying off	4	1.4
Special cows	3	1.1
Always questioned	3	1.1
Varying CSCC in test-day records in current lactation	2	0.7
Low CSCC in test-day records in current lactation and high last CSCC	2	0.7

CSCC = cow somatic cell count
[1] Farmers could indicate more than one reason

During the telephone interview, 245 farmers of the 292 farmers (84%) indicated to be interested in an additional diagnostic test. The reason of not being interested in any additional diagnostic test at drying off (47/292, 16%) was that they had enough information from existing sources such as SCC and electronic conductivity (36/47, 77%). Most interested farmers were indifferent about the test being at the cow or quarter

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level (177/245, 72%). Thirty-four farmers (14%) were only interested in a test at the cow level, and 34 farmers (14%) were only interested in a test at the quarter level. Farmers interested in a test at the cow level expressed that the test should indicate whether an antimicrobial treatment would be necessary (87/211, 41%). Furthermore, the test should preferably be cheap (16%), simple (10%), usable on-farm, quick, and reliable. If interested in a test at the quarter level, many farmers (138/211 65%) would consider applying DCT at the quarter level if a test fulfilling their requirements would be available. They considered using such a test as the next step to further reduce antimicrobial usage (88/211, 42%). Some farmers did not prefer a test at the quarter level because they expected such a test to be too expensive or too time consuming, or they referred to the interaction among quarters and therefore preferred to treat at the cow level.

In the field study, seven of the nine farmers indicated to be interested in an additional test at drying off. In addition to the above-mentioned characteristics, they indicated that the test should be legally approved for DCT decisions, and should provide new information. For all farmers, a low number of false negative results was more important than a low number of false positive results. Time to result was not considered very important. All farmers expected to use an additional test more often than they currently use labBC to base DCT decisions on, if such a test would meet their requirements.

All nine farmers participating in the field study incorporated CSCC of the last test-day record in their DCT decision-making. Other parameters used were CSCC over the current lactation, production at drying off, mastitis history of the current lactation, and health of the cow. LabBC results were usually not used for DCT decisions in the default situation, because the farmers experienced taking a milk sample as costly, time consuming, and laborious and because they nevertheless often obtained contaminated results.

In Table 4 the DCT decisions made by farmers are cross-tabulated by growth on labBC. For one cow the default decision differed at the quarter level and for two cows the BC decision differed at the quarter level (made by two different farmers).

Farmers indicated to question their default DCT decision for 19 out of the 73 participating cows (26%), because, for example, CSCC on last test-day record was above the cut-off, but still considered low (6 times), and high CSCC on the last test-day record but previous test-day records were low (5 times). Farmers decided to treat 13

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of these 19 cows with antimicrobials (68%). Farmers preferred to have had additional information available when they made the default DCT decision for 21 of 73 cows. The most frequently indicated information of interest was which antimicrobial to apply.

Farmers changed their DCT decision for 72 out of 287 quarters when labBC results were obtained. Changed decisions were most often from not using antimicrobials to using antimicrobials for reasons of high quarter SCC or growth on labBC. Based on the default decision, 146 of the 266 (55%) quarter treatments agreed with labBC results (contaminated labBC results were excluded) (Table 4). Including labBC results in the decision-making process increased the number of quarters that would have been treated in accordance with the labBC results to 174 (66% of the BC decisions). At the same time, however, it increased the number of quarters treated with antimicrobials. Remarkably, the percentage of quarters treated when labBC showed no growth was comparable for the default decision (22%) and for the BC decision (25%).

If the default DCT decision was applied at the cow level, 34 of the 60 cows were treated in agreement with labBC results of the composite samples (57%). If the BC decision would be applied at the cow level, 46 cows would have been treated in agreement with labBC results of the composite samples (77%).

Farmers indicated to question their BC decisions for 6 of the 73 cows. Reasons for being uncertain were that for example quarter SCC was high but labBC resulted in no growth, if growth on labBC was not from presumptive mastitis-causing bacteria, or if labBC showed growth but the cow was considered healthy. For 4 of these 6 cows farmers decided to apply antimicrobials (67%), of which one cow had no growth on labBC.

Sensitivity and Sp of the default decision at the quarter level was 0.62 and 0.43, respectively (Table 4). Sensitivity and NPV increased if labBC results were added to the decision-making process of farmers compared to the default decision. However, kappa remained fair for all decisions assessed. Compared to the default decisions, if labBC results were only used for high SCC cows (scenario a), Se dropped from 0.62 (default) to 0.30, NPV was similar, but antimicrobial usage dropped from 60% to 23%. Se of the default and BC decision at the cow level was similar to these decisions at the quarter level, as was antimicrobial usage. The percentage of incorrect decisions was lowest for BC decisions at the cow level.

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Table 4. Characteristics of different strategies for farmers to decide on dry-cow treatment compared to results of classical bacteriological culture using quarter samples or composite samples

		Growth on bacteriological culture							Effect of decisions	
Decision	**Antimicrobials**	**No**	**Yes**	**Se**	**Sp**	**PPV**	**NPV**	**kappa**	**Antimicrobial usage**	**Incorrect decisions[6]**
Quarter samples (*n* = 266)[1]										
Default decision[2]	No	44	63	0.62	0.43	0.64	0.41	0.33	0.60	0.45
	Yes	58	101							
BC decision[3]	No	35	25	0.85	0.34	0.67	0.58	0.28	0.77	0.35
	Yes	67	139							
Scenario a[4]	No	91	114	0.30	0.89	0.82	0.44	0.34	0.23	0.47
High CSCC BC decision, low CSCC untreated	Yes	11	50							
Scenario b[5]	No	33	25	0.85	0.32	0.67	0.57	0.29	0.78	0.35
Low CSCC BC decision, high CSCC treated	Yes	69	139							
Composite samples (*n* = 60)[1,6]										
Default decision	No	4	20	0.60	0.40	0.83	0.17	0.35	0.60	0.43
	Yes	6	30							
BC decision	No	5	9	0.82	0.50	0.89	0.36	0.27	0.77	0.23
	Yes	5	41							

Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value

[1] Contaminated results were excluded

[2] Dry-cow treatment decision made by farmers based on cow somatic cell count (CSCC) from test-day records and other cow and farm level information farmers normally use to make dry-cow treatment decisions

[3] Dry-cow treatment decision made by farmers based on default available information combined with bacteriological culture results at the quarter (quarter samples) or the cow level (composite samples)

[4] All low SCC cows (CSCC < 200,000 cells/mL milk) were hypothetically not dried off with antimicrobials and farmers made dry-cow treatment decisions based on bacteriological culture results for high SCC cows (CSCC ≥ 200,000 cells/mL)

[5] All high SCC cows were hypothetically dried off with antimicrobials and farmers made dry-cow treatment decisions based on bacteriological culture results for low SCC cows

[6] If the dry-cow treatment decision was to dry off ≥ 1 quarter with antimicrobials, the whole cow was assumed to be dried off with antimicrobials

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Discussion

This study aimed to determine the need of farmers for additional diagnostics on IMI at drying off, their interest in quarter or cow level DCT decision-making, and the effect of having labBC results available on DCT decisions made by farmers.

Farmers are aware that SCC is not a perfect test to diagnose an IMI and most farmers (84%) expressed a need for a diagnostic test in addition to CSCC and other available information to decide whether to apply antimicrobials at drying off. The percentage of farmers interested in such an approach was higher than expected and described earlier, when 31% of farmers was interested in such a test (Griffioen et al., 2016). This change may be caused by the fact that our earlier study was performed at a time when selective DCT was only recently introduced in the Netherlands. Nowadays, farmers are accustomed to selective DCT, realise they actually have to decide, and may have faced pitfalls when using CSCC to select cows for antimicrobial DCT. Most farmers indicated to sometimes question their DCT decision, especially if CSCC is in close proximity of cut-off values. Although multiple parameters are considered when a DCT decision is made (Scherpenzeel et al., 2016; this study), the combination of these parameters apparently still leads to uncertainty around DCT decisions. As farmers decided most often to dry off a cow with antimicrobials when uncertain, animals may be treated unnecessary. Including labBC results in the decision-making process decreased the total number of cows where farmers questioned their DCT decisions. Thus, incorporating bacteriological results in decision-making seems valuable to reduce uncertainty and to optimise antimicrobial use related to DCT because less cows would be treated unnecessary, which was the main reason for farmers to be interested in additional tests to improve DCT decisions.

Currently, farmers have used selective DCT for several years without affecting udder health in herds, and with reduced antimicrobial usage (Ekman and Østerås, 2003; Santman-Berends et al., 2016; Vanhoudt et al., 2018). Most selective DCT decisions are made at the cow level and only a minority of farmers sometimes decided at the quarter level. Applying selective DCT at the cow level is more protective against new IMI than applying selective DCT at the quarter level compared to blanket DCT (Robert et al., 2006; Halasa et al., 2009b). Furthermore, if farmers use labBC results of composite samples for cow level DCT decisions, the risk of an IMI at calving and the risk of clinical mastitis in the next lactation is similar as compared to blanket DCT (Cameron et al.,

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2014), However, most farmers indicated to be interested in an additional test at the quarter level, and to consider quarter level DCT decisions if such information would be available. The selective DCT decisions improved when farmers obtained the requested quarter level information, but still by far most DCT decisions were made at the cow level. Notable was the number of quarters with a negative labBC result for which the BC decision was to use antimicrobials. Although remarkable, it is in line with earlier findings of Neeser et al. (2006), who also described that mastitic quarters with negative culture results are treated with antimicrobials. One farmer mentioned to apply antimicrobials to labBC negative quarters because of SCC was high. Another explanation might be that farmers used other information for treatment decisions in addition to labBC results of a particular quarter, such as other quarters of a cow being culture positive. This hypothesis is in line with the remarks of farmers on interdependency of quarters of a cow. It does, however, contradict the indicated need of farmers to decide at the quarter level. Farmers obviously are interested in prudent as well as optimal antimicrobial use, but want to avoid risks on animal health, as described earlier by Scherpenzeel et al. (2016). Whether farmers will implement further steps to optimise selective DCT in the future, will also depend on factors such as regulations and social pressure (Lam et al., 2017). Whether selective DCT at the quarter level is the next step to optimise selective DCT compared to the current selective DCT approach should be further studied, and the economic consequences need to be calculated before such an approach should be advised.

A selective DCT program should be simple and inexpensive, and aiming to optimise antimicrobial use (Whist et al., 2006). Bacteriological culture results are important to optimise antimicrobial treatments as is emphasised in the Nordic countries (Ekman and Østerås, 2003), where numerous samples are submitted for labBC at drying off (Vilar et al., 2018), and as is experienced by farmers using on-farm culturing for cows with mastitis during lactation (Neeser et al., 2006). Our definition of an infected cow or quarter based on labBC results (one cfu cultured, regardless the type of pathogen) increased the Se of labBC to diagnose an IMI (Dohoo et al., 2011), reflected in the percentage of infected cows and quarters found. Compared to other studies, these percentages were similar at the cow level (Hawkins, 2019), or higher at both, the cow and quarter level (Cameron et al., 2014; Jashari et al., 2016; Gohary and McDougall, 2018). The definitions chosen have affected Se and Sp of the DCT decisions. However, the effect on treatment probably was limited, because most farmers made cow level DCT decisions and most cows had more than one labBC positive quarter. Furthermore,

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we only determined a quarter or cow as infected or uninfected. Including information on the aetiology of the IMI might improve udder health in the next lactation, although some debate exists on which pathogens can be left untreated at drying off (Berry and Hillerton, 2002; Østerås and Sølverød, 2009), Nevertheless, there might be potential to further optimise DCT decisions if the aetiology of the IMI is taken into account to direct DCT decisions. On the other hand, applying labBC to all cows is more time consuming and expensive and thus less practical than applying labBC after preselection of cows based on CSCC (Lam et al., 2009). Therefore, we evaluated the effect of selective using labBC results in making DCT decisions. Using culture results to decide on DCT for high CSCC cows (CSCC > 200,000 cells/mL) and leaving low CSCC cows untreated (scenario a) resulted in a very low antimicrobial usage where almost all treated cows were cows with an IMI (PPV of 0.8). However, Se was low, meaning that only 30% of the cows with an IMI was actually treated. Most of the untreated cows would also be left untreated according to the Dutch selective DCT guidelines based on the CSCC levels (Vanhoudt et al., 2018), without adverse effects on udder health (Santman-Berends et al., 2016). Depending on the goal a farmer has with DCT, the threshold of CSCC to select cows to sample for labBC might be lowered. That will increase Se, but will decrease Sp (Reksen et al., 2008; Jashari et al., 2016; Gohary and McDougall, 2018; Hawkins, 2019). Lower threshold levels will lead to increased sampling costs and to more quarters with a no growth result in labBC. These effects may result in farmers being less motivated to apply extra diagnostics in selective DCT. In addition, using the first scenario (a) resulted in a similar percentage of incorrect decisions, and a similar NPV compared to the default decision, with the latter being considered important by the farmers. Therefore, based on this study, the first scenario (a) seemed to be optimal because DCT decisions improved, and antimicrobial usage reduced compared to the default approach.

To conclude, farmers indicated to have a need for additional information on whether to apply antimicrobials at drying off, especially if CSCC is in close proximity to the indicated cut-off values for antimicrobial treatment. Bacteriological culture results added to the default available information used by farmers improved the quality of DCT decisions because the NPV increased. However, antimicrobial usage was higher too compared to the default approach. Although farmers indicated to be interested to dry off at the quarter level if a suitable test would be available, which might be the next step to further reduce antimicrobial use, they hesitated to do so. The selective use of labBC to decide on DCT for high SCC cows and leave low SCC cows untreated seemed the most

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optimal approach to optimise selective DCT decisions and to decrease antimicrobial usage as compared to the current approach.

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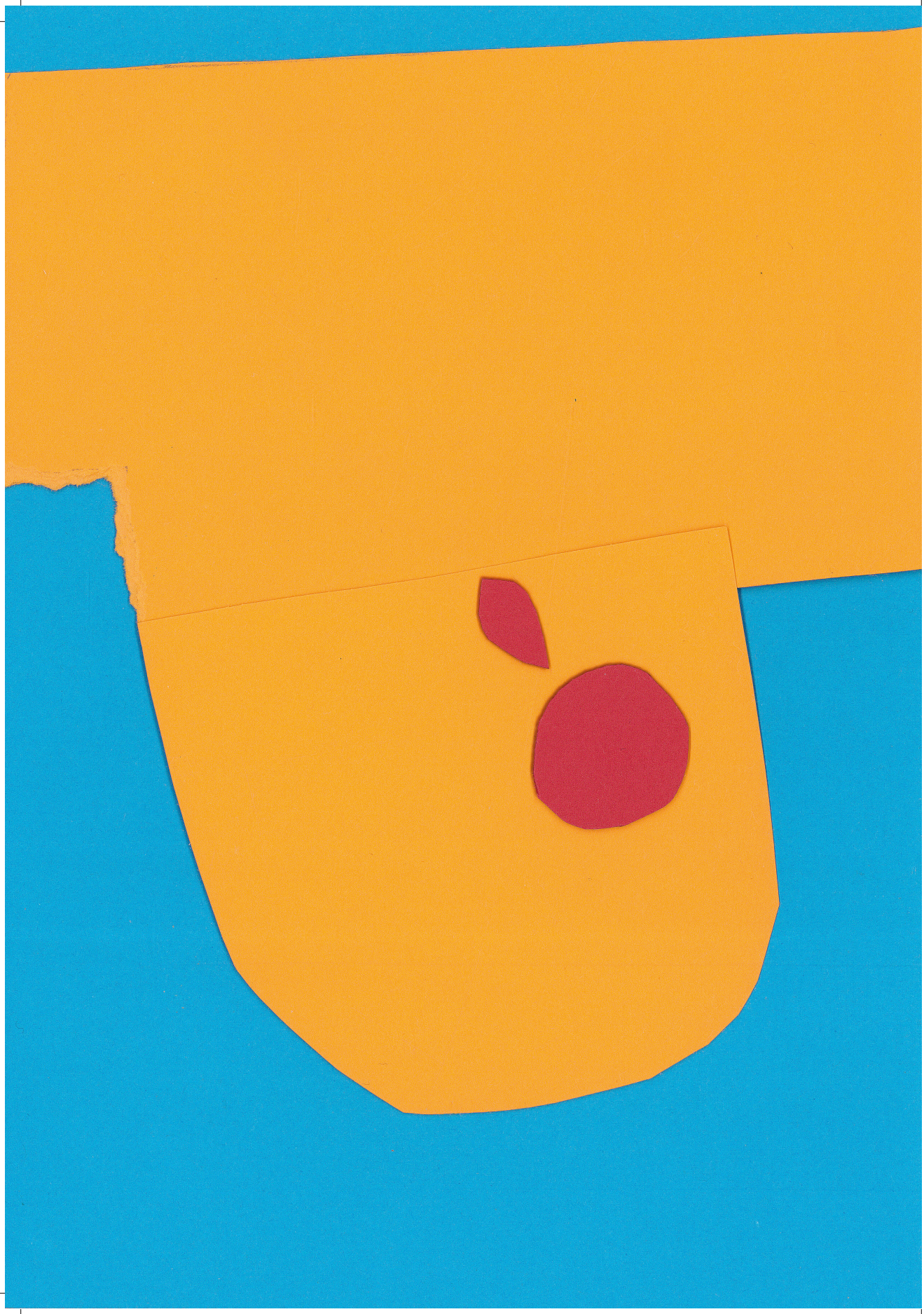

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The effect of a mastitis treatment strategy with or without on-farm testing

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Submitted for publication

Abstract

The etiology of an intramammary infection (IMI) is crucial information to use antimicrobials prudently in mastitis. This study aimed to evaluate the effect of two treatment strategies for cows with (sub)clinical mastitis, each using an on-farm test, on cure and antimicrobial use as compared to farmers' current treatment strategy. The two tests, CHROMagar Mastitis (CHROMagar, Paris, France) and Minnesota Easy Culture System II Tri-plate (University of Minnesota, St. Paul, MN, USA), were used by farmers and resulted in the etiology of the IMI. Two randomized controlled trials were conducted on 15 herds. Trial 1 prospectively enrolled 155 clinical mastitis cows that were evenly distributed over three groups: a test group using CHROMagar, a test group using Minnesota, and a control group not using on-farm tests. Trial 2 cross-sectionally included 78 subclinical mastitis cows, evenly distributed over the same three groups. In both trials, farmers decided on treatment based on the information they had, either 1 day after (both test groups; with prior treatment advise per test result), or on the day of enrollment (control group). The results of both trials showed that more treatments were in accordance with the cause of IMI if tests were used than without testing. For clinical mastitis, using CHROMagar resulted in lower intramammary antimicrobial usage, and using Minnesota resulted in antimicrobials used more targeted compared to the control group. For subclinical mastitis, treatment strategies with on-farm testing resulted in over 50% of the cows being treated with antimicrobials, compared to 4% in the control group. The bacteriological cure rate of cows with (sub)clinical mastitis was lower in the Minnesota group (odds ratio 0.24 (95% CI 0.06-0.90)), and of cows with clinical mastitis lower in the CHROMagar group (odds ratio 0.18 (95% CI 0.03-0.99)), compared to the control group.

Using on-farm tests in farmers' decisions-making process results in more targeted treatments than without on-farm testing. However, the similar or higher cure rates of farmers' current treatment strategy, suggests that farmers include important, predictive information in their treatment decision with respect to bacteriological cure. Further research is needed to determine how additional information, like on-farm test results and farmers' experience, can be combined in the treatment strategy of farmers to enhance prudent antimicrobial use and cure outcomes of cows with (sub)clinical mastitis.

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Introduction

Prudent use of antimicrobials in animals is key to minimize development of antimicrobial resistance. Nevertheless, antimicrobials should be available to treat animals in case of bacteriological infections. In dairy farms, most antimicrobials are used to treat mastitis (Pol and Ruegg, 2007; Kuipers et al., 2015). If farmers are able to identify the mastitis cases for which a treatment is likely to succeed, antimicrobials will be used more prudently.

Currently, farmers generally use clinical signs and mastitis history to decide upon antimicrobial treatment for mastitis cows (Owens et al., 1997; Vaarst et al., 2002; Sears and McCarthy, 2003). Clinical signs and mastitis history lack, however, the ability to inform on the etiology of the IMI (Ruegg, 2018). If the etiology of the IMI is known, farmers can target antimicrobial use (Pinzón-Sánchez et al., 2011). Bacteriological culture (BC) in a laboratory or on-farm is available to diagnose the bacteriological cause of an IMI and to support mastitis treatment decisions. Routine BC of milk samples in a laboratory, however, is only used sporadically by farmers (Griffioen et al., 2016; Kayitsinga et al., 2016). Nevertheless, farmers are interested in mastitis diagnostics and indicate they would use diagnostic tools more frequently if a quick, cheap, reliable, and preferably on-farm test would be available (Griffioen et al., 2016).

Various culture-based on-farm tests are available and seem to contribute to a more targeted mastitis treatment when employed under laboratory settings (McCarron et al., 2009; Royster et al., 2014; Ferreira et al., 2018). These on-farm tests are most often plates split multiple ways using chromogenic selective media or conventional selective media. In a previous study we evaluated four culture-based tests, two using chromogenic media (CHROMagar Mastitis (CHROMagar, Paris, France) (CHROMagar) and VétoRapid (Vetoquinol, 's-Hertogenbosch, the Netherlands)), and two using conventional selective media (Minnesota Easy Culture System II Tri-plate (University of Minnesota, St. Paul, MN, USA) (Minnesota) and Hardy Diagnostic Mastitis Tri-plate (Hardy Diagnostics, Santa Maria, CA, USA)) on milk samples routinely submitted to a laboratory for BC (Griffioen et al., 2018). We concluded that all tests agree more or less equally with BC to indicate IMI. Ferreira et al. (2018) evaluated four tests too, and found that the chromogenic test Accumast (FERA Animal Health LCC, Ithaca, NY), has a higher accuracy compared to BC than the tests using conventional media, being Minnesota, and the Mastitis Quad plates SSGN and SSGNC (both DQCI Services, Mounds View, MN).

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These evaluations were all done in a laboratory environment where the tests were not read by farmers.

In an US study it was found that most mastitis treatment decisions made by farmers using the Minnesota Easy Culture Bi-plate, a test with conventional selective media, agree with those that would be taken if the results would have been based on BC results. However, what the effect is of chromogenic or conventional selective culture-based tests used on-farm by farmers on treatment decisions is unknown. It has been described that if a treatment strategy is based on culture results and thus treatment is delayed 24 h, bacteriological cure rate of clinical mastitis is equal to a situation in which all cows are treated immediately with the same antimicrobials (Lago et al., 2011; Vasquez et al., 2017). In both studies, mastitis cases with Gram-negative results and with no growth culture results were not treated with antimicrobials whereas Gram-positive results were treated with antimicrobials. Consequently, antimicrobial usage was reduced when a test was used to determine treatment, opposed to treating all mastitis cows (Lago et al., 2011; Vasquez et al., 2017). However, also without the use of a culture-based test in practice, not all clinical mastitis cases are treated with antimicrobials (Vaarst et al., 2002; Santman-Berends et al., 2016). Furthermore, subclinical mastitis is hardly treated during lactation, and farmers do indicate to have a need for on-farm tests to decide whether or not to treat those cases (Griffioen et al., 2016). To our knowledge, the effect of treatment strategies as applied by farmers after on-farm tests were used on antimicrobial use and on cure of clinical and subclinical mastitis, has never been compared to a control group where farmers treated cows using their current decision-making process, without having information on the etiology of the IMI available.

In this study, we recorded treatment decisions made by farmers either using an on-farm test, CHROMagar or Minnesota, or using their current treatment decision strategy, often based on clinical signs or SCC, and compared their decisions to the treatments we would advise based on results of BC performed in a laboratory. The aim of this study was to evaluate the effect of two treatment strategies of cows with clinical and subclinical mastitis, in which farmers used either of 2 on-farm tests as compared to the current treatment strategy farmers apply on (1) antimicrobial use, (2) quantitative antimicrobial usage, (3) bacteriological cure, (4) quarter SCC (QSCC), (5) intramammary cure (defined as the combination of bacteriological cure and low QSCC), (6) new IMI, and, in case of clinical mastitis, (7) clinical cure of the affected quarter.

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Materials and Methods

Study design

Two randomized controlled clinical trials were conducted in 15 commercial Dutch dairy herds. Trial 1 evaluated two on-farm culture-based mastitis tests, CHROMagar and Minnesota, to be used for treatment decisions for cows with clinical mastitis (Figure 1). Trial 2 evaluated the same two tests to be used for treatment decisions for cows with subclinical mastitis (Figure 2).

Farm selection

Farmers were eligible to participate in both trials if they milked at least 100 dairy cows, participated in the routine milk recording program (CRV, the Netherlands), and had a conventional management (organic farmers were excluded, farmers with automatic milking systems were allowed to participate). Farmers were recruited through veterinarians within the authors' network or directly through the network of the authors.

Trial 1 – Clinical Mastitis

Fifteen farmers started between May 2017 and March 2018 in trial 1 in which 155 cows were enrolled with clinical mastitis. Enrollment stopped in the period May – July 2018. The study protocol, aseptical sample collection, and on-farm culturing and interpretation of test results were explained to the farmer by study personnel during a farm visit at the start of trial 1. Farmers were asked to include cows with grade 1 clinical mastitis (mild mastitis, only abnormal milk) or grade 2 clinical mastitis (moderate mastitis, abnormal milk and quarter affected). Cows were ineligible to participate if they had participated before, had clinical mastitis in multiple quarters, had been treated with antimicrobials during the last 30 d, or were scheduled to be dried off within 21 d after diagnosing mastitis. Eligible cows were randomly assigned to the on-farm test group or the control group by the farmer by taking a numbered envelope. Half of the participating farmers started with an on-farm test group using CHROMagar and the other half started using Minnesota. After half the anticipated number of clinical mastitis cases was enrolled on a farm, the farmer switched to use the other test. The protocol for the control group was unchanged. We aimed for three evenly distributed groups by assigning two-thirds of the mastitis cows to the on-farm test groups and one-third to the control group. Of all cows in the trial, farmers aseptically collected one milk sample of the affected quarter on d 0. The sample was immediately frozen on farm.

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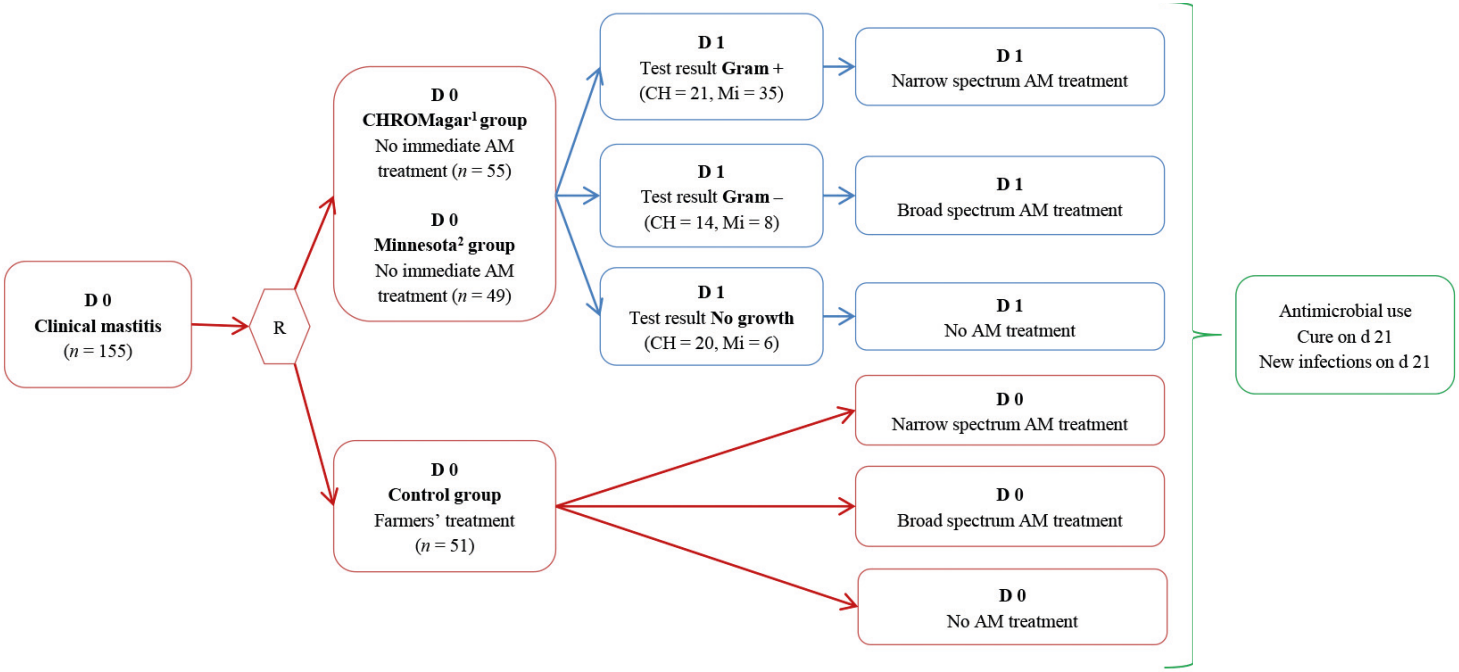

Figure 1. Study design of trial 1. The number of cows with clinical mastitis in 1 quarter that were randomly assigned (R) to one of two on-farm test groups using Minnesota Tri-plate or CHROMagar Mastitis, or to the control group by picking an envelope by the farmer. Two-thirds of the cows were assigned to the on-farm test groups, where a treatment strategy was applied in which the farmer used the on-farm test on d 1 and one-third to the control group where the farmer applied his currently used treatment strategy. The farmer used one of the on-farm mastitis tests for half of the anticipated number of clinical mastitis cases during trial 1. After that, the farmer used the other on-farm mastitis test on clinical mastitis cases that were enrolled during the remainder of trial 1. Proportion of cure, new intramammary infections and antimicrobial usage were assessed on d 21 for all cows. [1]CHROMagar Mastitis (CHROMagar, Paris, France; CH), [2]Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA; Mi), AM = antimicrobial

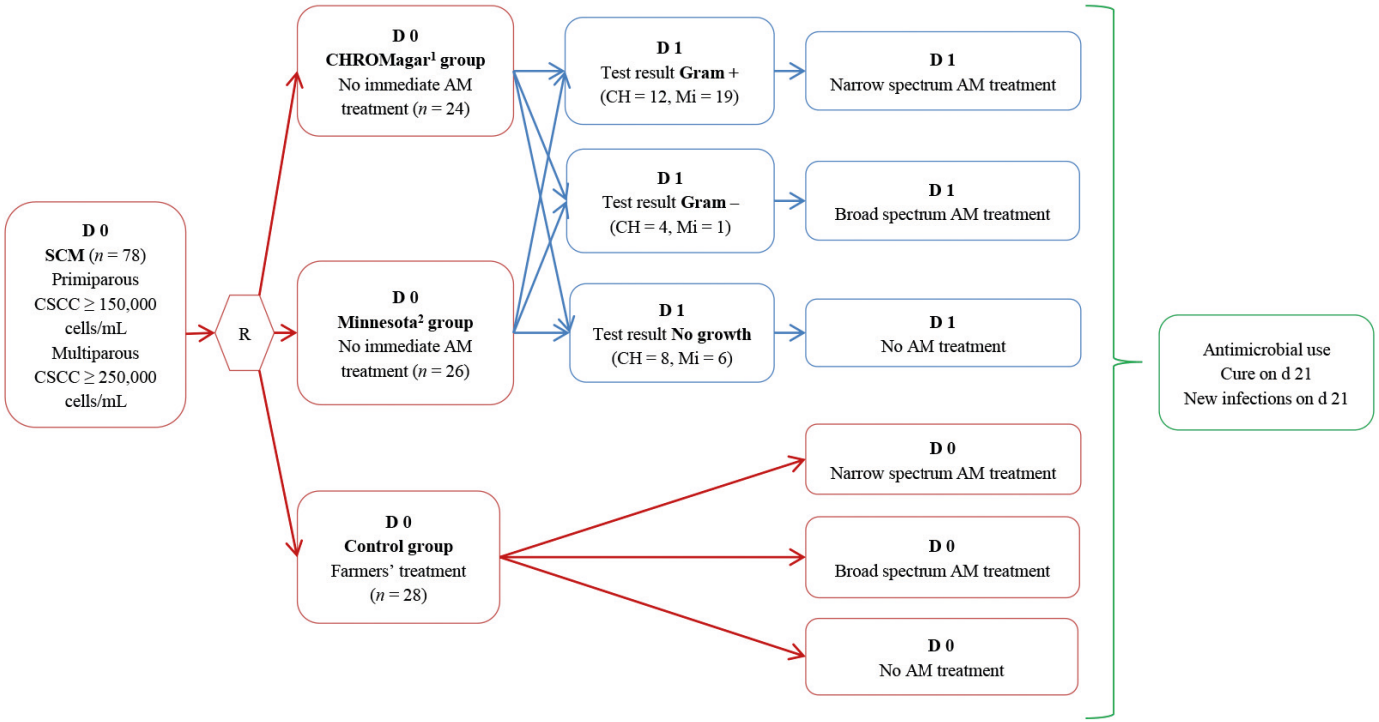

Figure 2. Study design of trial 2. The number of cows with subclinical mastitis in one quarter that were randomly assigned (R) to one of three groups: the CHROMagar group, or the Minnesota group, both where a treatment strategy was applied in which the farmer used the on-farm test on d 1, or to the control group where the farmer applied his currently used treatment strategy. Proportion of cure, new intramammary infections and antimicrobial usage were assessed on d 21 for each of the three groups. [1]CHROMagar Mastitis (CHROMagar, Paris, France; CH), [2]Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA; Mi), AM = antimicrobial

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A second milk sample was taken from the quarters in the on-farm test groups at the same time to be cultured by the farmer using the on-farm mastitis test. In the control group, farmers treated the cows the way they were used to, without testing. On d 21 another milk sample was collected by farmers from all quarters enrolled in the trial, which was also immediately frozen on farm. The frozen samples were collected by study personnel regularly to be cultured in a veterinary laboratory using routine BC. Farmers were asked to record date and symptoms of all quarters with clinical mastitis that were enrolled in the study, and to classify severity of the mastitis as grade 1, grade 2, or grade 3 (severe mastitis, abnormal milk, quarter affected, and systemic signs of illness) on d 0. Additionally, farmers were asked to record milk yield on d 0, whether the cow had clinical mastitis earlier in the current lactation, and for cows in the control group, the treatment that was applied. For cows in the on-farm test groups, the farmers recorded the intended treatment if no on-farm test would have been used on d 0, and the on-farm test result and the treatment applied on d 1. Furthermore, farmers were asked to record any treatment given to the included cows during the first 21 d after clinical mastitis as well as whether clinical signs in the affected quarter were present on d 21. If after the initial treatment a secondary antimicrobial treatment was given within 21 d, the d 21 sampling date was postponed to 9 d after the last antimicrobial treatment.

Trial 2 – Subclinical Mastitis

Trial 2 started on the final enrollment day of trial 1 in each of the 15 herds, in the period May to July 2018. Seventy-eight cows with subclinical mastitis were enrolled. The study protocol was explained to the farmer at the start of trial 2. On-farm culturing, interpretation of test results, and aseptical sample collection were done the same way as in trial 1. Cases of subclinical mastitis were selected by the study personnel, based on the most recent test-day record. Subclinical mastitis was defined as high cow SCC (CSCC) (primiparous cows CSCC ≥ 150,000 cells/mL and multiparous cows CSCC ≥ 250,000 cells/mL (de Haas et al., 2008, CRV, 2019)). The same exclusion criteria applied as in trial 1. In addition, cows that had calved within 5 d before the test-day record were excluded, as well as cows nominated to be sold within 30 d. The sampling day was scheduled within 10 d after the test-day of the milk recording. Samples were collected by the farmer or by study personnel. The California Mastitis Test (CMT) was used to identify quarters with elevated SCC in selected high CSCC cows. Trace to +++ were considered positive CMT results. Cows with only one CMT positive quarter were eligible for enrollment, as were cows in which abnormal milk was observed in one quarter. The latter cows were considered to have clinical mastitis and were included in trial 1. A milk sample was taken

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aseptically of the CMT positive quarter. Study personnel randomly assigned eligible cows evenly into the CHROMagar group, the Minnesota group, and the control group. For cows in the on-farm test groups, the collected milk sample was cultured on farm on the day of sampling (d 0). On d 1 the farmer read and interpreted the results and treated the cow using the test result. In the control group, cows were treated the way the farmer would otherwise do without testing. All collected samples were transported on ice to the laboratory and frozen at – 20 °C until BC. A subsequent milk sample (d 21) was collected aseptically by study personnel or the farmer from all quarters enrolled in trial 2. If collected by the farmer, these samples were frozen on farm and transported later to the laboratory, if collected by study personnel, samples were immediately transported on ice to the laboratory and were frozen at – 20 °C until BC. As in trial 1, farmers were asked to record milk yield, mastitis history during the current lactation, and the treatment applied to cows in the control group on d 0. For cows in the on-farm test groups, the farmers recorded the intended treatment if no on-farm test would have been used on d 0, and the on-farm test result and the treatment applied on d 1. Any treatment given after the initial treatment was also recorded by the farmers up to d 21. If a secondary treatment was given within 21 d, d 21 sample collection was postponed until 9 days after the end of treatment.

Follow up

After the trial was over, farmers were asked whether or not they were willing to continue with a treatment strategy for mastitis including an on-farm test. If they did not express that intention they were asked why. The farmers that were willing to continue were asked to score their willingness to use an on-farm test on a 0 (never) to 5 (always) scale, for the different grades of mastitis, and which of the on-farm tests they preferred.

Routine bacteriological culture of milk

All frozen milk samples were thawed at room temperature in the bacteriological laboratory of Royal GD Animal Health (Deventer, the Netherlands). Milk samples were bacteriologically cultured, and SCC of the sample was determined using fluorescence flow cytometry (CombiScope 600, Delta Instruments, Drachten, the Netherlands) (ISO 13366-2|IDF 148-2:2006, 2006). For BC, 0.01 mL of milk was inoculated onto 6% sheep blood agar (Biotrading, Mijdrecht, the Netherlands). Growth of presumptive mastitis-causing bacteria was examined after incubation for 18 to 24 h at 37 °C (under aerobic conditions) and again 24 h later. Species identification of presumptive mastitis-causing pathogens was performed by matrix-assisted laser desorption ionization time-of-flight

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mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper Microflex LT (Bruker Daltonics GmbH, Germany) (Barreiro et al., 2010). Milk samples that tested negative with the standard culture procedure and had a SCC above 200,000 cells/mL were cultured again onto sheep blood agar following a combination of freezing and pre-incubation (Sol et al., 2002). An IMI was defined as a pure culture or predominance of one or two types of presumptive mastitis-causing pathogens with ≥ 1 colony forming unit (cfu) on the plate. The presence of *Staphylococcus aureus*, *Streptococcus agalactiae*, and hemolytic streptococci, was always considered as an IMI. No growth of presumptive mastitis-causing pathogens was defined as no growth. Contamination was defined as growth of more than two phenotypically different colony types, without a dominant presumptive mastitis-causing pathogen. Bacteriological growth was categorized into one of four diagnostic categories, being Gram-positive growth, Gram-negative growth, no growth or contamination as described by Griffioen et al. (2018). Readers of BC were blind to the intervention groups and to the on-farm tests results. Results of BC were provided to the farmers after trial 2 ended.

On-farm culture-based mastitis tests

CHROMagar Mastitis

CHROMagar consists of two Petri dishes each with a different agar. One agar is specific for Gram-positive bacteria (with peptone and yeast extract, salt and a chromogenic mix) and one is specific for Gram-negative bacteria (with peptone and yeast extract and a chromogenic mix). For the use of CHROMagar a manual was drawn up in Dutch, based on the available documents online (CHROMagar Mastitis version 2, 2014), which was provided to the farmers to be used for plating and interpreting CHROMagar. In short, the farmer mixed the milk sample gently, dipped a sterile cotton swab in the milk for 8 to 10 seconds to become fully saturated and plated the milk onto the Gram-positive plate. A new sterile cotton swab was then dipped in the milk for 8 to 10 seconds and streaked onto the Gram-negative plate. Both agars were incubated at 37 °C for 18 to 24 h. Growth of ≥ 1 cfu on the Gram-positive plate was considered Gram-positive growth, growth of ≥ 1 cfu on the Gram-negative plate was considered Gram-negative growth. No growth on both plates was defined as no growth.

Minnesota Easy Culture II Tri-plate

Minnesota consists of one Petri dish split in three separate sections (triplate). One section is specific for Gram-positive growth (factor agar), one for Streptococcal growth (modified TKT agar), and one for Gram-negative growth (MacConkey agar). The English

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manual was translated into a Dutch manual and provided to the farmers to be used for plating and interpreting Minnesota. In short, the farmer mixed the milk sample gently, dipped a sterile cotton swab in the milk for 8 to 10 seconds to become fully saturated. The milk was streaked onto the factor agar, dipped in the milk, streaked onto the MacConkey agar, dipped again and streaked onto the modified TKT agar. Minnesota was incubated at 37 °C according to the manufacturer's guidelines for 18 to 24 h (Minnesota Easy Culture System User's Guide, 2013). Growth of ≥ 1 cfu on the factor agar was considered Gram-positive growth, with or without growth on TKT agar. Growth of ≥ 1 cfu on the MacConkey agar was considered Gram-negative growth. No growth on the Petri dish was interpreted as no growth.

Treatment

For cows in the on-farm test groups in both trials a treatment strategy was applied in which the on-farm test results as obtained by the farmer were included. A Gram-positive test result was advised to be treated with a narrow spectrum intramammary antimicrobial effective against Gram-positive bacteria (Formularium, 2016). A Gram-negative test result (regardless of the presence of Gram-positive growth) was advised to be treated with a broad spectrum intramammary antimicrobial effective against Gram-positive and Gram-negative bacteria (there is no approved narrow spectrum antimicrobial effective against Gram-negative bacteria available in the Netherlands) (Formularium, 2016). If the test result was no growth the farmer was advised to not treat the quarter with an antimicrobial. For cows in the control group, farmers decided on the type of treatment the way they were used to, based on the farm specific treatment plan, where treatments are categorized based on clinical signs and CSCC. In this group, cows could also be treated with either a narrow or a broad spectrum intramammary antimicrobial or with no antimicrobial at all. Other treatments than antimicrobial treatments were possible.

Statistical analysis

Baseline characteristics

Baseline characteristics of the three intervention groups were compared using a *t*-test to evaluate equality between the intervention groups for both trials. Differences were considered statistically significant if $P < 0.05$.

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Test characteristics

Results of the on-farm tests as interpreted by the farmers in both trials were compared to the results of BC to calculate the test characteristics of the two on-farm mastitis tests. Sensitivity and specificity, with the accompanying binominal exact 95% CI, were calculated for Gram-positive growth, Gram-negative growth and no growth as described by Dohoo et al. (2009). Furthermore, the proportion of agreement between BC and the on-farm tests corrected for chance was calculated, known as Cohen's kappa. The guideline of Landis and Koch (1977) was used to determine the magnitude of agreement. A value of > 0.80 was considered to be almost perfect, > 0.60-0.80 substantial, > 0.40-0.60 moderate, > 0.20-0.40 fair, > 0.00-0.20 slight, and ≤ 0.00 poor agreement.

Treatment decisions

For both trials, farmers' treatment decisions were compared to the advised treatment given the on-farm test result (agreeOF) in both on-farm test groups and compared to decisions that would be taken if BC results were used (agreeBC) in all three intervention groups. AgreeOF and agreeBC were positive if a case with Gram-positive growth on the on-farm test respectively on BC was treated with narrow spectrum intramammary antimicrobials, if a case with Gram-negative growth on the on-farm test respectively on BC was treated with broad spectrum intramammary antimicrobials, and if a case with no growth on the on-farm test respectively on BC was not treated with antimicrobials (Formularium, 2016).

Proportions of cure

Outcomes were evaluated on the group level, irrespective of whether or not an antimicrobial treatment was given, for the three intervention groups in both trials combined on d 21 after enrollment. Outcomes determined were bacteriological cure, low QSCC (< 100,000 cells/mL in d 21 sample), intramammary cure (combination of the previous two), and new IMI of the affected quarter. For trial 1, also clinical cure was determined. Bacteriological cure of a quarter was defined as the original bacterial species cultured from the d 0 milk sample not being cultured by BC from the d 21 sample. For this part of the analysis quarters with no growth or quarters with contaminated d 0 samples were excluded. A quarter was considered to have a new IMI when a bacterial species was cultured from the d 21 sample that was not cultured from the d 0 sample. Quarters with contaminated d 0 samples were excluded from this part of the analysis. Clinical cure was defined as the absence of clinical signs on d 21 as recorded by the farmers.

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Logistic regression models

Multivariable logistic regression analysis was performed for all cows with mastitis in the study to determine effects of different explanatory variables on cure using Stata Statistical Software: Release 15 (StataCorp LLC College Station, TX, USA). The explanatory variables tested were the intervention groups (control group, CHROMagar group, or Minnesota group), severity of mastitis (subclinical mastitis, grade 1 clinical mastitis, grade 2 clinical mastitis), etiology of the IMI on d 0, d 0 milk production (continuous), parity (1, 2, 3, or > 3), DIM on d 0 (≤ 100 d or > 100 d), whether CSCC on the last test-day record prior to d 0 was low or high (for subclinical mastitis this was the test-day record prior to the test-day record used for selection of cows). Farmer was included as random effect. The intervention group of the cow was always forced into the models. Via stepwise backward selection, insignificant variables were excluded 1 by 1 until a model was obtained including the intervention group, and variables significant in the model (including confounders: variables resulting in a change in beta-coefficients of other important variables of ≥ 20% when removed from the model). Significant variables were identified by comparing the goodness of fit (log-likelihood) of the models using Akaike's Information Criterion (a difference of > 2 was considered significant).

We anticipated the enrollment of 80 cases of mastitis in each intervention group. Based on the formula

$$N = \frac{2p\,(100 - p)}{\Delta^2}\,[Z\,(1 - \alpha) + Z\,(1 - \beta)]^2$$

(Jones et al., 1996) we would be able to show an effect when the difference in bacteriological cure between the groups would be ≥ 18% with a power of 80% (β) and a CI of 95% (α, 1-tailed). We set the probability of bacteriological cure (p) at 70% (Lago et al., 2011), and Δ is the difference between two groups.

6

Results

Participating farmers and cows

Trial 1 started with 12 farmers, of which two stopped participating shortly after the start of the trial. One farmer stopped because she was unwilling to delay treatment of clinical mastitis for 18 – 24 h and one farmer because he struggled with record keeping. Data on these two herds were not included in the study. In March 2018, five additional

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herds were enrolled, because the number of mastitis cases was lower than anticipated, resulting in a total of 15 herds. Eight farmers started using Minnesota in the on-farm test group and seven started using CHROMagar. The average time farmers participated in the study was 279 d (106 d – 394 d). Herds were spread over the middle of the Netherlands with four herds located in the province of Flevoland, four in Overijssel, three in Gelderland, and one herd in each of the provinces Utrecht, Drenthe, Noord-Brabant, and Zuid-Holland. Five farmers milked with an automatic milking system and ten with a milking parlor. Participating herds had on average 150 lactating cows (99 – 270), an average milk production of 9,847 kg per year (8,200 – 11,000 kg per year), and an average bulk tank SCC of 168,429 cells/mL (90,000 – 325,000 cells/mL).

All farmers indicated they were able to culture and interpret the on-farm test according to the instructions given, and appreciated the additional information provided by the test results. Some farmers hesitated to postpone treatment for 24 h for the first enrolled clinical mastitis cases, but they became more secure after experiencing that severity of mastitis was not affected by a delayed treatment. Moreover, some farmers left cows untreated if they considered them to be clinically cured after 24 h, even if the on-farm test showed growth. After the trial, 11 farmers indicated they were willing to continue to use an on-farm test. Four farmers could not decide or did not intend to use the tests in the future, because the time to result was too long, or because they had gained too little experience with working with the tests. The interested farmers preferred to use a test most for grade 2 clinical mastitis (scored on average 4.1), followed by subclinical mastitis (on average 3.7), grade 1 mastitis (on average 3.0), and grade 3 mastitis (on average 3.0). Seven farmers preferred CHROMagar and five Minnesota, whereas three farmers could not indicate their preference.

A total of 233 mastitis cases were enrolled in the study over both trials (Table 1). For trial 1, 163 quarter samples were collected on d 0. Of these quarters, eight had missing d 21 samples and were therefore excluded from the analysis, resulting in 155 cows included in trial 1. For trial 2, 315 cows were selected as subclinically infected cows based on CSCC on the last test-day record. Of these cows, 90 were ineligible to participate because of recent antimicrobial treatment or because they were nominated to be sold shortly, 138 had either zero or two or more quarters with a positive CMT result, and were for that reason not included in the study. Three cows were found to have signs of clinical mastitis and were therefore included in trial 1. After enrollment six cows were excluded by lack of d 21 samples. In total 78 cows with 1 CMT positive

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quarter fitted the inclusion criteria, and were included in trial 2. In total over both trials, 79 cases were enrolled in the CHROMagar group, 75 cases in the Minnesota group, and 79 cases in the control group. The total number of enrolled cases per farm varied from 2 to 39. Two farmers only used CHROMagar during the study, the other farmers used each of both tests at least once. In trial 1, one farmer had not enrolled any case of clinical mastitis and one farmer only one case. In 1 herd, none of the selected subclinical mastitis cows was eligible for participation in trial 2. In another herd, all d 21 samples of the subclinical quarters were lacking, and this herd was therefore excluded from trial 2. Characteristics of the three intervention groups in both trials are shown in Table 1. Farmers categorized eight cows as grade 3 mastitis. Although we advised not to include grade 3 mastitis cows, we kept these cows in the analysis. We considered these as grade 2 mastitis cows, because based on the recorded symptoms only two of them had systemic signs of illness, whereas of the cows categorized as grade 2 mastitis, 13 had systemic signs of illness too. Additionally, waiting 1 day before treatment was not considered to be an issue by the farmers. Five of the grade 3 mastitis cows were in the CHROMagar group, one in the Minnesota group, and two in the control group. In trial 1, 15 cows had experienced clinical mastitis earlier in the same lactation, of which four in the CHROMagar group, eight in the Minnesota group, and three in the control group. In trial 2, five cows had experienced clinical mastitis earlier in the same lactation, of which two in the CHROMagar group, three in the Minnesota group, and none in the control group. In trial 2, the average DIM was significantly higher in the Minnesota group than in both other groups.

Test results

The results of BC are shown in Table 2. Almost 20 percent of all milk samples were culture negative in the laboratory. Significantly more Gram-positive bacteria (mainly Staphylococci), and significantly less Gram-negative bacteria were cultured from clinical mastitis cases in the Minnesota group than in the control group. No other significant differences were found. Table 3 shows the test characteristics of CHROMagar and Minnesota as interpreted by the farmers compared to the results of BC. Minnesota had a higher agreement with BC for all three diagnostic categories than CHROMagar.

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Table 1. Average number of cases per farm and characteristics of cows with clinical mastitis or subclinical mastitis at enrollment for two on-farm test groups where on-farm test results were used by farmers to determine treatment, and for the control group where cows were treated as the farmer was used to without testing

	Clinical mastitis			**Subclinical mastitis**		
	On-farm test group			**On-farm test group**		
	CHROMagar[1]	**Minnesota[2]**	**Control[3]**	**CHROMagar**	**Minnesota**	**Control**
Cases (*n*)	55	49	51	24	26	28
Average number of cases per farm (min.-max.)	3.7 (0-9)	3.3 (0-8)	3.4 (0-9)	1.6 (0-6)	1.7 (0-5)	1.9 (0-6)
Average days in milk (min.-max.)	120 (0-460)	138 (-9-354)	136 (4-411)	139 (22-338)[a]	215 (24-737)[b]	122 (11-255)[a]
Average parity	3.5 (1-8)	3.0 (1-9)	3.3 (1-7)	3.7 (1-8)	2.7 (1-5)	3.3 (1-7)
Median quarter SCC Day 0 (x 1,000 cells/mL)[4]	-	-	-	1011	1076	1125
Median cow SCC -1 test-day record before event (x1,000 cells/mL)[5]	163	93	76	271	319	233
Median cow SCC -2 test-day record before event (x1,000 cells/mL)[6]	129	79	76	109	305	128
Average milk production Day 0 (kg)	32.9	32.7	34.6	35.6	32.7	36.5
Average milk production -1 test-day record before event (kg)[5]	37.5	36.2	36.7	36.0	34.0	36.4
Average milk production -2 test-day record before event (kg)[6]	36.1	37.7	36.2	34.8	33.0	38.8

[a,b] indicates significant difference between groups for that variable using a *t*-test with $P < 0.05$ defined as statistically significant

[1] On-farm test group using CHROMagar Mastitis results (CHROMagar, Paris, France) on-farm to determine intramammary infection status and treatment accordingly

[2] On-farm test group using Minnesota Easy Culture System II Tri-plate results (University of Minnesota, St Paul, MN, USA) on-farm to determine intramammary infection status and treatment accordingly

[3] Control group where no mastitis test was used. Cases were treated as the farmer was used to, based on the farm specific treatment plan

[4] As determined in d 0 milk sample

[5] As determined on last test-day record prior to d 0

[6] As determined on second last test-day record prior to d 0

Table 2. Quarter level etiology of IMI according to routine bacteriological culture as cultured from d 0 milk samples from 155 clinical mastitis cases and from 78 subclinical mastitis cases stratified over two on-farm test groups where on-farm test results were used by farmers to determine treatment, and a control group where cows were treated as the farmer was used to, based on the farm specific treatment plan without testing

	Cultured bacteria trial 1						Cultured bacteria trial 2					
	On-farm test group						On-farm test group					
	CHROMagar (*n* = 55)		Minnesota (*n* = 49)		Control (*n* = 51)		CHROMagar (*n* = 24)		Minnesota (*n* = 26)		Control (*n* = 28)	
	n	fraction	*n*	fraction	*n*	fraction	*n*	fraction	*n*	fraction	*n*	fraction
1 colony morphology	41	0.75	41	0.84	37	0.73	17	0.71	20	0.77	17	0.61
2 colony morphologies[1]	3	0.05	1	0.02	3	0.06	1	0.04	0	0.00	6	0.21
≥ 3 colony morphologies	0	0.00	0	0.00	0	0.00	0	0.00	2	0.08	1	0.04
No growth	11	0.20	7	0.14	11	0.22	6	0.25	4	0.15	4	0.14
Gram-positive bacteria	36	0.65	37	0.76	26	0.51	18	0.75	17	0.65	25	0.89
Staphylococcus spp.	9	0.16	16	0.33	12	0.24	13	0.54	9	0.35	13	0.46
S. aureus	4	0.07	7	0.14	5	0.10	3	0.13	2	0.08	0	0.00
NAS	5	0.09	9	0.18	7	0.14	10	0.42	7	0.27	13	0.46
Streptococcus spp.	16	0.29	15	0.31	9	0.18	1	0.04	5	0.19	4	0.14
S. uberis	8	0.15	8	0.16	4	0.08	0	0.00	1	0.04	0	0.00
S. agalactiae	0	0.00	0	0.00	0	0.00	1	0.04	2	0.08	1	0.04
S. dysgalactiae	7	0.13	7	0.14	5	0.10	0	0.00	1	0.04	2	0.07
Other Gram-positive bacteria	7	0.13	3	0.06	2	0.04	2	0.08	3	0.12	8	0.29
Enterococcus spp.	4	0.07	3	0.06	3	0.06	3	0.13	0	0.00	0	0.00
Gram-negative bacteria	11	0.20	6	0.12	17	0.33	1	0.04	3	0.12	4	0.14
Lactose fermenting bacteria	11	0.20	5	0.10	16	0.31	0	0.00	0	0.00	0	0.00
Escherichia coli	9	0.16	4	0.08	14	0.27	0	0.00	0	0.00	0	0.00
Klebsiella spp.	1	0.02	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Other coliform bacteria[2]	1	0.02	1	0.02	2	0.04	1	0.04	3	0.12	4	0.14
Other Gram-negative bacteria	0	0.00	1	0.02	1	0.02	0	0.00	0	0.00	0	0.00

[1] Up to two bacterial species were differentiated if present in a milk sample
[2] *Serratia* spp., *Enterobacter* spp., and *Citrobacter* spp.

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Treatment decisions

The treatment decision of the farmer differed after an on-farm test was used from the treatment that would have been applied if no on-farm test was used for 53% of the cows, in both trials together. Most of the different intended and applied treatments were for cows with clinical mastitis, where approximately 60% of the decisions changed after an on-farm test was used. For clinical mastitis, using CHROMager resulted most often in decisions that changed from using antimicrobials to not using antimicrobials (45% of the changed decisions), whereas using Minnesota resulted most often in decisions that changed from using narrow spectrum antimicrobials to using broad spectrum antimicrobials (41% of the changed decisions). Using Minnesota, the smallest percentage of changed decisions was for using antimicrobials in the intended treatment to not using them in the applied treatment (24% of the changed decisions). For cows with subclinical mastitis, 30% (Minnesota) to 46% (CHROMagar) of the intended and applied treatments differed. Almost all decisions for subclinical mastitis changed from not using antimicrobials to using antimicrobials.

Table 3. Sensitivity (Se), specificity (Sp), and agreement (kappa) of two on-farm tests, CHROMagar Mastitis (CHROMagar, Paris, France) and Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA), used by farmers on milk samples collected from cows with clinical and subclinical mastitis as compared to routine bacteriological culture

	CHROMagar (*n* = 79)			**Minnesota (*n* = 75)**		
Result	**Se (95% CI)**	**Sp (95% CI)**	**kappa (95% CI)**	**Se (95% CI)**	**Sp (95% CI)**	**kappa (95% CI)**
Gram-positive growth	0.57	0.68	0.20	0.91	0.59	0.52
	0.46-0.68	0.58-0.78	0.11-0.29	0.84-0.97	0.48-0.70	0.41-0.64
Gram-negative growth	0.67	0.85	0.42	0.56	0.94	0.49
	0.56-0.77	0.77-0.93	0.31-0.53	0.44-0.67	0.89-0.99	0.38-0.61
No growth	0.47	0.69	0.12	0.45	0.89	0.33
	0.36-0.58	0.59-0.80	0.05-0.19	0.34-0.57	0.82-0.96	0.23-0.44

In trial 1, the percentage of clinical mastitis cows treated with an antimicrobial was lowest in the CHROMagar group (Table 4). If Minnesota was used to decide on treatment, 77% of the antimicrobial treatments was a narrow spectrum antimicrobial. In both other groups approximately 50% of the antimicrobials given were narrow spectrum intramammary antimicrobials. Furthermore, less systemic antimicrobials were given to cows in the Minnesota group. In trial 2, the proportion of cows treated with an antimicrobial was higher in the on-farm test groups than in the control group.

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Almost 100% of the antimicrobial treatments given in trial 2 were narrow spectrum intramammary antimicrobial treatments. Over both trials, 57% of the treatments was positive for agreeBC in the CHROMagar group, this was 67% of the treatments in the Minnesota group, and 32% of the treatments in the control group. Furthermore, farmers followed the on-farm test result in their treatment decision in most cases, which is resembled in the percentage of treatments positive for agreeOF, being 90% of the treatments in trial 1 and 70% of the treatments in trial 2. In situations in which the test result was not followed in both trials, generally no antimicrobials were applied although bacteriological growth was found on the on-farm test. The three groups in both trials were equal with respect to the percentage of quarters dried off, of secondary treatments, and of replacement (data not shown).

Cure of mastitis

The bacteriological cure rate was lower for cows with (sub)clinical mastitis if farmers applied a treatment strategy using Minnesota (odds ratio 0.24) compared to the treatment strategy applied in the control group (Table 5). In this model, CSCC on the last test-day record, parity, and milk production were also needed in the model, but had no effect on the association of the treatment strategy with bacteriological cure. Milk production and CSCC were found to have a confounding effect on the association of cows with parity > 3 and bacteriological cure. For intramammary cure and low QSCC, no differences were found between the three groups. In both models, severity of mastitis was found to have a confounding effect on the association of the treatment strategy with both cures.

Cows with clinical mastitis that were assigned to the CHROMagar group had a lower bacteriological cure rate (odds ratio 0.18) than cows assigned to the control group (Table 6). In this model, CSCC on the last test-day record was needed in the model, but had no effect on the association of the treatment strategy with bacteriological cure. For intramammary cure and low QSCC, no differences were found between the three groups. In both models, severity of mastitis was found to have a confounding effect on the association of the treatment strategy with both cures. For clinical cure, CSCC on the last test-day record was found to be a confounder of the effect of the treatment strategy on the probability of clinical cure.

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Table 4. Treatments applied to cows with clinical mastitis and subclinical mastitis in two on-farm test groups where the on-farm test CHROMagar Mastitis or Minnesota Tri-plate was used by farmers to determine treatment, and in the control group where cows were treated as the farmer was used to, without testing

	Clinical mastitis						**Subclinical mastitis**					
	On-farm test group						**On-farm test group**					
	CHROMagar[1]		**Minnesota[2]**		**Control[3]**		**CHROMagar**		**Minnesota**		**Control**	
	***n*/total**	***n* fraction**	***n*/total**	***n* fraction**	***n*/total**	***n* fraction**	***n*/total**	***n* fraction**	***n*/total**	***n* fraction**	***n*/total**	***n* fraction**
Antimicrobial use	32/55	0.58	39/49	0.80	44/51	0.86	13/24	0.54	13/26	0.50	1/28	0.04
Intramammary	32/32	1.00	39/39	1.00	44/44	1.00	13/13	1.00	13/13	1.00	1/1	1.00
Narrow spectrum[4]	17/32	0.53	30/39	0.77	23/44	0.52	13/13	1.00	11/13	0.85	1/1	1.00
Broad spectrum[5]	15/32	0.47	9/39	0.23	21/44	0.48	0/13	0.00	2/13	0.15	0/1	0.00
Systemic	15/32	0.47	12/39	0.31	18/44	0.41	3/13	0.23	4/13	0.31	0/1	0.00
No antimicrobial treatment	23/55	0.42	10/49	0.20	7/51	0.14	11/24	0.46	13/26	0.50	27/27	0.96
Alternative treatment[6]	7/23	0.30	3/10	0.30	4/7	0.57	1/11	0.09	3/13	0.23	2/27	0.07
Treatment agreed with the on-farm test result[7]	45/55	0.82	44/49	0.90	-	-	17/24	0.71	18/26	0.69	-	-
Treatment agreed with BC result[8]	30/55	0.55	37/49	0.76	19/51	0.37	15/24	0.63	13/26	0.50	6/28	0.21
Total	55/155	0.35	49/155	0.32	51/155	0.33	24/78	0.31	26/78	0.33	28/78	0.36

[1] On-farm test group using CHROMagar Mastitis (CHROMagar, Paris, France) results on-farm to determine intramammary infection status and treatment accordingly
[2] On-farm test group using Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA) results on-farm to determine intramammary infection status and treatment accordingly
[3] Control group where cases were treated as the farmer was used to, based on the farm specific treatment plan
[4] Narrow spectrum intramammary antimicrobial specific against Gram-positive bacteria
[5] Broad spectrum intramammary antimicrobial against both Gram-positive and Gram-negative bacteria
[6] An alternative treatment was applied like anti-inflammatory drugs or udder mint, but no antibiotics were applied
[7] Treatment agreed if Gram-positive on-farm test results were treated with a narrow spectrum antimicrobial, if Gram-negative on-farm test results were treated with a broad-spectrum antimicrobial, and if no growth on-farm test results were not treated with an antimicrobial.
[8] Treatment agreed if Gram-positive growth on routine bacteriological culture was treated with a narrow spectrum antimicrobial, if Gram-negative growth was treated with a broad-spectrum antimicrobial and if no growth was not treated with an antimicrobial

Table 5. Logistic regression model for the odds of intramammary cure, bacteriological cure, low QSCC, and new IMI of all participating cows with mastitis determined on d 21 after sample collection. The treatment strategy group was forced into the models. Farm was included as random effect

		Intramammary cure[1] (*n* = 121)		Bacteriological cure[2] (*n* = 121)		Low QSCC[3] (*n* = 181)		New IMI (*n* = 142)	
		OR	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
Treatment strategy group[4]	Control	Referent		Referent		Referent		Referent	
	CHROMagar	1.15	0.37-3.57	0.29	0.08-1.05	1.86	0.69-5.01	0.73	0.30-1.76
	Minnesota	0.94	0.30-2.93	0.24	0.06-0.90	1.72	0.64-4.62	0.59	0.24-1.48
Grade of mastitis[5]	0	Referent		-		Referent		-	
	1	1.23	0.30-4.99	-		1.39	0.47-4.15	-	
	2	5.40	1.61-18.07	-		4.62	1.72-12.41	-	
Parity	1	-		Referent		-		-	
	2	-		6.16	1.32-28.75	-		-	
	3	-		4.49	1.09-18.58	-		-	
	>3	-		1.58	0.42-5.89	-		-	
Cow SCC test-day record -1[6]	Low	-		Referent		-		-	
	High	-		0.14	0.05-0.37	-		-	
Milk production	Continuous	-		1.05	1.00-1.10	-		-	
Constant		0.11	0.03-0.40	1.28	0.16-10.09	0.08	0.03-0.26	0.52	0.28-0.96

[1] Quarter was positive for bacteriological cure and had a low QSCC
[2] The colony forming unit cultured from the d 0 sample was not cultured from the d 21 sample (no growth and contaminated d 0 samples were excluded)
[3] Quarter SCC (QSCC) in d 21 sample < 100,000 cells/mL
[4] 1) Control group where cases were treated as the farmer was used to, without testing; 2) Test group using CHROMagar Mastitis (CHROMagar, Paris, France) results on-farm to determine treatment; 3) Test group using Minnesota Easy Culture System II Triplate (University of Minnesota, St Paul, MN, USA) results on-farm to determine treatment
[5] Coded as 0 = subclinical mastitis, 1 = grade 1 mastitis, 2 = grade 2 mastitis
[6] Cows SCC (CSCC) as recorded on the last test-day record prior to d 0 (low CSCC was defined for primiparous cows < 150,000 cells/mL and for multiparous cows < 250,000 cells/mL)

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Table 6. Logistic regression model for the odds of intramammary cure, bacteriological cure, low QSCC, new IMI, and clinical cure of cows with clinical mastitis determined on d 21 after mastitis detection. The treatment strategy group was forced into the models. Farm was included as random effect

		Intramammary cure[1] (*n* = 83)		**Bacteriological cure[2] (*n* = 83)**		**Low QSCC[3] (*n* = 118)**		**New IMI (*n* = 97)**		**Clinical cure (*n* = 110)**	
		OR	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
Treatment strategy group[4]	Control	Referent		Referent		Referent		Referent		Referent	
	CHROMagar	0.83	0.24-2.88	0.18	0.03-0.99	1.04	0.34-3.19	0.88	0.31-2.51	1.43	0.42-4.90
	Minnesota	0.65	0.18-2.30	0.23	0.04-1.36	1.36	0.45-4.10	1.10	0.34-3.19	2.53	0.61-10.56
Grade of mastitis	1	Referent		-		Referent		-		-	
	2	4.13	1.31-12.98	-		3.12	1.20-8.10	-		-	
Cow SCC test-day record -1[5]	Low	-		Referent		-		-		Referent	
	High	-		0.15	0.05-0.48	-		-		0.14	0.05-0.43
Constant		0.18	0.05-0.64	25.58	4.95-132.02	0.16	0.05-0.52	0.45	0.22-0.96	7.53	2.62-21.64

[1] Quarter was positive for bacteriological cure and had a low QSCC
[2] The colony forming unit cultured from the d 0 sample was not cultured from the d 21 sample (no growth and contaminated d 0 samples were excluded)
[3] Quarter SCC (QSCC) in d 21 sample < 100,000 cells/mL
[4] 1) Control group where cases were treated as the farmer was used to, without testing; 2) Test group using CHROMagar Mastitis (CHROMagar, Paris, France) results on-farm to determine treatment; 3) Test group using Minnesota Easy Culture System II Triplate (University of Minnesota, St Paul, MN, USA) results on-farm to determine treatment
[5] Cows SCC (CSCC) as recorded on the last test-day record prior to d 0 (low CSCC was defined for primiparous cows < 150,000 cells/mL and for multiparous cows < 250,000 cells/mL)

Discussion

This study aimed to evaluate the effect of two treatment strategies for cows with (sub)clinical mastitis, each using an on-farm test, on cure and antimicrobial use as compared to farmers' current treatment strategy. Our study showed that the current treatment strategy of farmers resulted in similar or higher bacteriological cure than when on-farm tests were incorporated in their decision-making process. However, if a treatment strategy including on-farm test results is used, mastitis treatment decisions become more targeted, because two to three times more treatment decisions were in accordance with the cause of the IMI in the on-farm test groups than in the control group.

Also in human studies it has been described that diagnostic interventions often fail to improve patient outcomes as in only 18% of the compared studies patient benefits were reported, even though diagnostic tests performed well (Siontis et al., 2014). Additionally, a study that determined the effect of a culture-based treatment program on bacteriological cure of clinical mastitis in cows also reported a lower, although non-significant, odds ratio for bacteriological cure in the culture-based treatment group (odds ratio 0.6) compared to the control group where all cows were treated with antimicrobials (Lago et al., 2011). In that study, over 80% of the decisions were in accordance with bacteriological culture results from the laboratory if an on-farm test was applied. We also found a lower bacteriological cure rate if an on-farm test was used as compared to if no test was used to decide on treatment for (sub)clinical mastitis, with a high percentage of treatments in accordance with the cause of the IMI. The odds for bacteriological cure of (sub)clinical mastitis were found to be highly affected by cow factors like CSCC on the last test-day record. For example, bacteriological cure rates were low if an on-farm test was used to decide on treatment for a cow with clinical mastitis that also had a high CSCC on the last test-day record. Apparently, farmers include that type of information in their treatment strategy, which it is not reflected in the result of an on-farm mastitis test, but is of high value to be incorporated in the decision-making process to enhance cure probabilities (Kromker and Leimbach, 2017; Ruegg, 2018). Thus, cow factors that affect bacteriological cure should be considered before an antimicrobial treatment is applied.

As farmers indicated to be interested in using on-farm tests for subclinical mastitis, we studied the effects on cure in these cases too. If farmers used on-farm tests in their

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decision-making process for subclinical mastitis, more treatments were in accordance with the cause of the IMI as compared to the control group, but antimicrobial usage increased from almost 0% to over 50%. We found that cows in the 2nd or 3rd parity with (sub)clinical mastitis with a low CSCC on the last test-day record, and therefore likely recently infected, have high bacteriological cure probabilities, irrespective of the treatment strategy used. Although for recently infected cows with subclinical mastitis treatment might be considered, the effect on bacteriological cure should outweigh the negative effect of increased antimicrobial usage. Although most of the cows with subclinical mastitis were left untreated in the control group, bacteriological cure was as good as in the groups in which on-farm tests were used. As we were unable to show a beneficial effect of using an on-farm test in the treatment strategy on bacteriological cure of subclinical mastitis cases, the use of on-farm tests is not advised. If farmers want to treat cows with subclinical mastitis, thorough examination of the (sub)clinical mastitis history of the cow should be performed (Kromker and Leimbach, 2017). In addition, the etiology of the IMI should be incorporated in the decision-making process because cure probabilities differ with etiology (Ruegg, 2018). Further research should be performed to determine how to incorporate the results of on-farm tests in the selection of cows with high cure probabilities to enhance optimal and prudent treatment of cows with subclinical mastitis.

If farmers decide on mastitis treatment, they will add the information from test results to the information they usually use, like history of clinical mastitis or history of CSCC from the current lactation (Vaarst et al., 2002; Neeser et al., 2006). Consequently, not only test characteristics affect cure, but also the way test results are incorporated in treatment decisions (Siontis et al., 2014). In our study, farmers followed the on-farm test results in most clinical mastitis treatment decisions (82-90%), and thus just over half of the decisions changed after the on-farm test was used as compared to the intended treatment. Cases where the on-farm test result was not followed, often were culture positive results that were left untreated. These may have been cows that already were clinically cured during the 24 h pending test results. As a consequence, by the time test results became available, farmers felt no urge to treat these cows anymore. This may be due to self-cure of mastitis as earlier described (Ruegg, 2018). Some farmers seemed to be unaware of this phenomenon, as we noticed farmers initially hesitated to postpone treatment. However, after experiencing the apparent self-cure, some farmers left these cows untreated, even though bacterial growth was detected on the on-farm test. A minority of the farmers already was familiar with the possible self-cure of cows

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and anticipated on that by a delayed decision on treatment of cows in the control group. This type of delayed treatment while watching the cow closely in the meantime has earlier been described as 'watchful waiting' (Ruegg, 2018), and may in part explain that not all cows in the control group in trial 1 were treated. Even though the treatments were more targeted, cure was not found to be improved after using an on-farm test. We checked whether strictly applying only treatments that were accordance of on-farm test results would have resulted in an improved cure, but found no positive effect (data not shown). Farmers may need to gain experience using on-farm tests, specifically on how to incorporate the test results into their current treatment decision-making process in order to enhance prudent use of antimicrobials for mastitis.

Farmers were able to work with both on-farm tests evaluated and had no specific preference for either of the two tests. Culturing milk samples of cows with mastitis is advised to determine targeted treatment for mastitis (Formularium, 2016; Lago and Godden, 2018; Ruegg, 2018). Although in our study the improved treatments lacked the ability to improve cure, we found that using on-farm tests for cows with clinical mastitis, can help farmers to improve antimicrobial use in three ways. First, using on-farm tests may lead to lower usage of antimicrobials in clinical mastitis because cases that might not benefit from an antimicrobial treatment, like culture negatives, can be selected. In our study in both on-farm test groups in trial 1, antimicrobials were less often applied to cows with culture negative BC results (11% of the culture negative cows in the CHROMagar group, 8% in the Minnesota group), compared to the control group (22% of the culture negative cows). Furthermore, if cows with Gram-negative on-farm test results would have been left untreated too, 69.1% of the clinical mastitis cases would not have received antimicrobials, which is comparable to the 68.5% reported in other studies where these cases were left untreated (Lago et al., 2011; Vasquez et al., 2017). Therefore, determining the etiology of IMI with an on-farm test can help to apply antimicrobials targeted at cases that likely benefit from it and thus to reduce antimicrobial usage in cases that do not. Second, on-farm tests help farmers to use less systemic antimicrobials, and therefore to apply antimicrobials more prudently. If Minnesota was used, the percentage of cows treated with antimicrobials was reduced slightly. However, the percentage of cows that received systemic antimicrobials was reduced considerably (24% in the Minnesota group compared to 35% in the control group), which improves prudent antimicrobial use as parenteral mastitis treatments are less targeted than intramammary treatments (Hillerton and Berry, 2005) and may have other collateral effects on antimicrobial resistance. Third, applying on-farm tests might

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help farmers to postpone treatment and thus to decrease antimicrobial usage. Although some farmers feel the urge to treat clinical mastitis immediately, treatment of clinical mastitis can be postponed 24 h without affecting clinical cure (Kromker and Leimbach, 2017; Vasquez et al., 2017). In this study, by forcing farmers to postpone treatment, they experienced that a delayed treatment is possible. Therefore, using on-farm tests might help farmers to get confidence in the watchful waiting approach, postponing treatment, which allows cows the possibility to self-cure IMI. Thus, both tests can be used by farmers in an on-farm environment, to differentiate IMI in three broad diagnostic and therapeutic categories in a quick way and to better target treatment decisions.

In conclusion, farmers are able to work with the two mastitis tests on-farm. Incorporating the results in their treatment decisions-making process results in more targeted treatments than if farmers decide on treatment without using on-farm test results. However, the similar or higher cure rates of farmers' current treatment strategy, suggests that farmers include important, predictive information in their treatment decision with respect to bacteriological cure. Therefore, further research is needed to determine how additional information, like on-farm test results and farmers' experience, can be combined in the treatment strategy of farmers to enhance prudent antimicrobial use and cure outcomes of cows with (sub)clinical mastitis.

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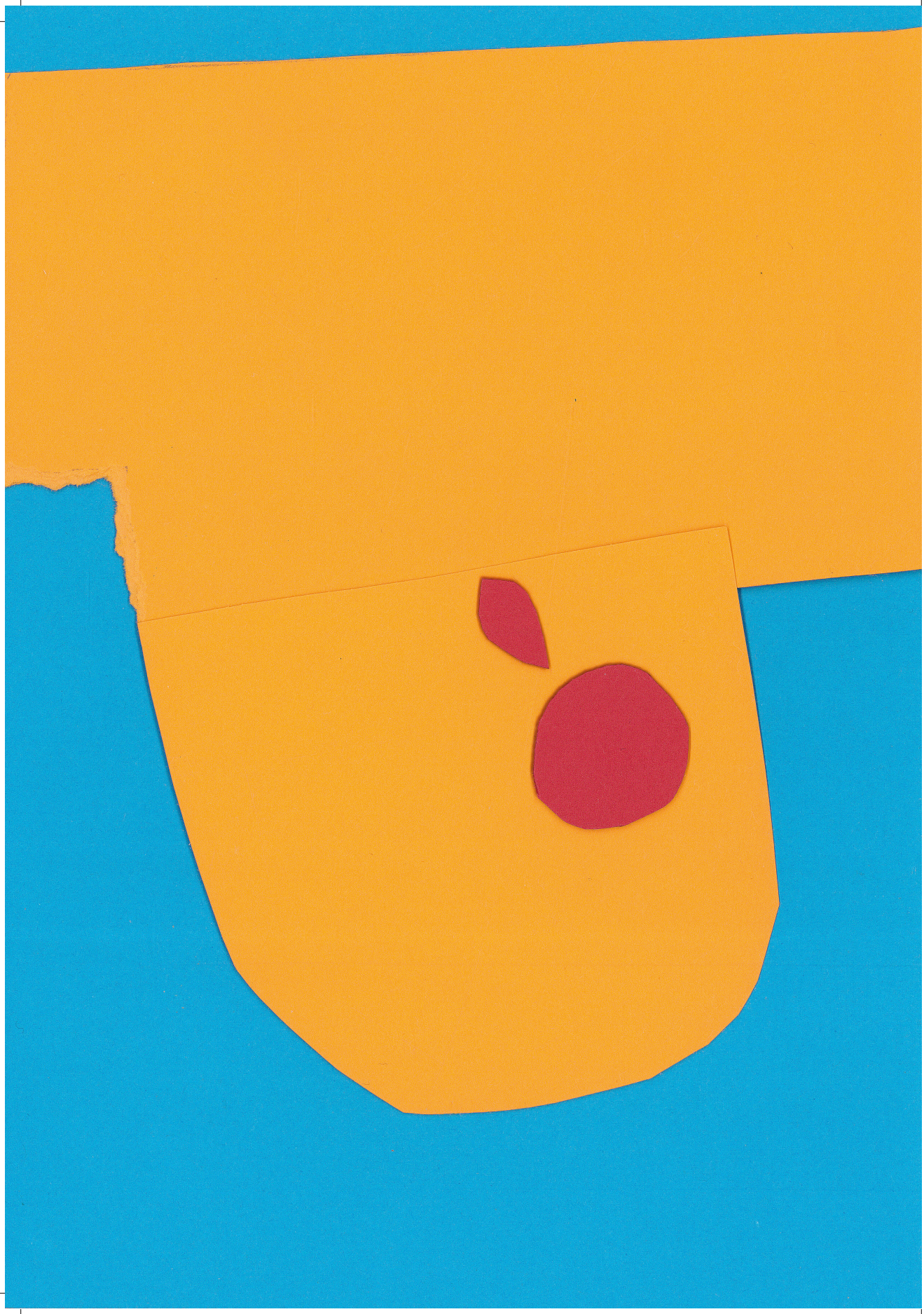

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Comparing on-farm tests and routine bacteriological culture to detect mastitis-causing pathogens in bovine milk samples using latent class analysis

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In preparation

Abstract

Bacteriological culturing is an important part of diagnostics in mastitis management. There is, however, no perfect test available to determine whether mastitis-causing pathogens are present in milk samples. Therefore, sensitivity (Se) and specificity (Sp) of new tests, such as on-farm tests, cannot be estimated without bias because of misclassification of the reference test. Given the increased use of this type of tests, these test characteristics are of importance. Through the use of Bayesian latent class analysis, the unknown status of samples can be modeled, and test characteristics can be estimated. In diagnostic tests, aspects such as inoculum volume, experience of the reader, and storage conditions of samples likely are important for the outcome. Therefore, the aim of this study was to estimate Se and Sp of two mastitis tests, CHROMagar Mastitis (CHROMagar, Paris, France) and Minnesota Easy Culture System II Tri-plate (University of Minnesota, St. Paul, MN, USA), used on-farm by farmers and used in the laboratory. Results were compared with standard bacteriological culturing (BC) executed in a laboratory, to identify three latent statuses: presence of Gram-positive bacteria, Gram-negative bacteria, or 'no growth' (i.e. no mastitis-causing bacteria) in milk samples. Mastitis milk samples collected in 15 dairy herds were cultured on farm and in the laboratory with CHROMagar (n = 79) and with Minnesota (n = 75), and with BC (n = 154) in the laboratory. We estimated Se and Sp of all tests and the prevalence of the different statuses in each of the 15 herds with a Bayesian latent class model. All tests had Se estimates ≥ 0.90 to detect Gram-positive bacteria, except CHROMagar used on-farm. Therefore, if farmers would use an on-farm test to determine treatment against Gram-positive bacteria, Minnesota would be preferred.

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Introduction

Mastitis has a major contribution to the total antimicrobial usage on dairy farms (Pol and Ruegg, 2007; Kuipers et al., 2015). For prudent antimicrobial use in mastitis treatments, knowledge on the presence of potential causative Gram-positive or Gram-negative bacteria in milk samples seems of utmost importance (Roberson, 2012). Although such information can be obtained through bacteriological culture (BC) of milk samples performed in a laboratory, farmers rarely use this type of information (Griffioen et al., 2016; Kayitsinga et al., 2016). To facilitate targeted treatments of mastitis, on-farm tests to detect mastitis-causing pathogens have been developed that shorten the time to result, by omitting transport of milk samples to the laboratory (Hiitiö et al., 2015; Ganda et al., 2016; Leimbach and Krömker, 2018). Various on-farm tests to detect mastitis-causing pathogens involved with intramammary infection (IMI) have been evaluated, using BC as a reference test (Mansion-de Vries et al., 2014; Royster et al., 2014; Viora et al., 2014). In a study evaluating BC itself, sensitivity (Se) and specificity (Sp) of BC were estimated at 0.86, and 0.75 respectively to detect any pathogen as compared to a pseudo gold standard (Dohoo et al., 2011b). For treatment of mastitis we are mainly interested in 3 statuses being the presence of Gram-positive, Gram-negative, or no bacteria in the milk sample (Krömker and Leimbach, 2017). For these statuses, however, no Se and Sp estimates are available leading to a non-specified misclassification of the reference test and thus to biased estimates of the evaluated on-farm tests. An often used and proven effective method to deal with imperfect reference tests is through latent class analysis (LCA) (Enøe et al., 2000). Latent class analysis models an unknown disease status, and estimates the test characteristics of the tests under evaluation in combination with the prevalence of the latent status in two or more distinct populations. This approach has previously been used to determine test characteristics of laboratory mastitis tests (Cederlöf et al., 2012; Mahmmod et al., 2013b) and of on-farm tests (Jones et al., 2019). As the performance of a test is also influenced by the reader, test characteristics should preferably be determined in the setting of intended use (Abuelo and Alves-Nores, 2016). Therefore, in this study, two on-farm tests, CHROMagar Mastitis (CHROMagar, Paris, France) and Minnesota Easy Culture System II Tri-plate (University of Minnesota, St. Paul, MN, USA) were used on farm as well as in the laboratory. The aim of this study was to estimate Se and Sp of CHROMagar and Minnesota on farm and in the laboratory to identify Gram-positive bacteria, Gram-negative bacteria, or 'no growth' in milk samples.

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Materials and methods

Two randomized controlled clinical trials were conducted in 15 commercial dairy herds between May 2017 and July 2018 as described in *Chapter 6*, and will be briefly described here. In that study, the effect of treatment strategies with or without on-farm testing on cure and antimicrobial use for mastitis was assessed. Trial 1 prospectively enrolled 104 cows with clinical mastitis that were evenly distributed over two groups: a group using CHROMagar on-farm (CHROMFARM**)**, and a group using Minnesota on-farm (MINFARM**)**. Trial 2 cross-sectionally included 50 cows with subclinical mastitis, evenly distributed over the same two groups. Subclinical mastitis was defined as a high cow somatic cell count (CSCC) (for primiparous cows CSCC ≥ 150,000 cells/mL, for multiparous cows CSCC ≥ 250,000 cells/mL) on a selected test-day record (de Haas et al., 2008), with one quarter being positive in the Californian Mastitis Test. In trial 1, a cross-over design was used: eight farmers started using Minnesota and seven started using CHROMagar. After half the anticipated number of clinical mastitis cases was enrolled, the farmers switched to use the other test. In trial 1, farmers aseptically collected two quarter milk samples from all participating cows; one sample was immediately frozen on farm, the other sample was used to inoculate the on-farm test the farmer worked with at that point in time. In trial 2, one quarter milk sample was collected from each cow, which was randomly assigned to inoculate one of the two on-farm tests and was thereafter frozen at – 20 °C. The frozen samples were collected regularly from the farms and were all cultured in the laboratory using the same test that was used on-farm (CHROMLAB, or MINLAB). In addition, these samples were subjected to standard BC.

For BC, 0.01 mL of milk was inoculated onto 6% sheep blood agar (Biotrading, Mijdrecht, the Netherlands). Growth of presumptive mastitis-causing bacteria was examined after incubation for 18 to 24 h at 37 °C (under aerobic conditions) and again 24 h later. Species identification of presumptive mastitis-causing pathogens was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using the MALDI Biotyper Microflex LT (Bruker Daltonics GmbH, Germany) (Barreiro et al., 2010). Milk samples that tested negatively with the standard culture procedure and had a somatic cell count above 200,000 cells/mL were cultured again onto sheep blood agar following a combination of freezing and pre-incubation to increase sensitivity (Sol et al., 2002). A positive result for BC was defined as a pure culture or predominance of one or two types of presumptive mastitis-causing pathogens with ≥ 1 colony forming unit (cfu) on the plate. The presence of *Staphylococcus aureus*, *Streptococcus agalactiae*, and hemolytic

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streptococci, was always considered as a positive result. 'No growth' was defined as the absence of growth of presumptive mastitis-causing pathogens. Results of BC were categorized into one of three diagnostic categories, being Gram-positive growth, Gram-negative growth, or 'no growth' as described by Griffioen et al. (2018). Farmers cultured and interpreted CHROMFARM and MINFARM. For that, a manual was provided to the farmers which was based on the available online documents for CHROMagar (CHROMagar Mastitis version 2, 2014), and the English manual for Minnesota (Minnesota Easy Culture System User's Guide, 2013). In short, for CHROMagar, consisting of two separate Petri dishes, the farmer used a sterile cotton swab saturated with milk to streak the milk onto the Gram-positive and the Gram-negative plate. Both plates were incubated at 37 °C for 18 to 24 h. Gram-positive growth was defined as bacterial growth of ≥ 1 cfu on the Gram-positive plate. Gram-negative growth was defined as bacterial growth of ≥ 1 cfu on the Gram-negative plate. 'No growth' was defined as no bacterial growth on both plates. The Minnesota test is a Tri-plate, consisting of a factor agar, a MacConkey agar, and a TKT agar. The farmer used a sterile cotton swab saturated with milk to streak the milk onto the three segments before Minnesota was incubated at 37 °C for 18 to 24 h. Gram-positive growth was defined a bacterial growth of ≥ 1 cfu on the factor agar, with or without growth on TKT agar. Gram-negative growth was defined as bacterial growth of ≥ 1 cfu on the MacConkey agar. 'No growth' was defined as no bacterial growth on either of the agars. In the laboratory, the same manuals were used as on farm. CHROMLAB and MINLAB were inoculated and interpreted by one of two people, one with and one without experience reading these plates, and BC was performed by experienced laboratory technicians. Readers of all tests were blind to the results of the other tests.

We evaluated three latent statuses: 'no growth', i.e. no culturable mastitis-causing bacteria present in the milk sample, Gram-positive, i.e. presence of Gram-positive bacteria in the milk sample, and Gram-negative, i.e. presence of Gram-negative bacteria in the milk sample. Three Bayesian latent class models were built to determine Se and Sp of the five tests under comparison (CHROMFARM, CHROMLAB, MINFARM, MINLAB, and BC) and the prevalence for the each of the three latent statuses per farm. Results of CHROMagar and Minnesota, were considered to originate from separate populations, resulting in 30 populations in total. For all populations BC results were available for prevalence estimation, whereas for 15 populations CHROMagar results were available and for 15 populations Minnesota results. An example of this model design is shown in Krogh et al. (2011). The results of all tests were tabulated as being positive or negative

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for each of the three latent statuses in R 3.2.3 (R Core Team, 2015). Bayesian LCA was performed using OpenBUGS version 3.2.3 (Lunn et al., 2009). Three Markov Chain Monte Carlo (MCMC) chains with different initial values were ran simultaneously for 10,000 iterations with the first 1,000 iterations being discarded as burn-in. Convergence was evaluated by visual inspection of the MCMC trace plots after the burn-in phase. If needed to obtain convergence, we set a lower limit for Se parameters (> 0.15) and Sp parameters (> 0.30).

In Bayesian analysis, prior information can be included in the model, based on available data. For the tests we compared, however, no prior information was available, because there are no studies that estimated Se and Sp for CHROMagar, whereas studies that estimated Se and Sp for Minnesota used BC as a perfect reference test (Royster et al., 2014, Ferreira et al., 2018). Although Se and Sp for BC were estimated with LCA (Mahmmod et al., 2013b, Jones et al., 2019), these did not use the same latent statuses as we did, that are at least necessary to determine antimicrobial treatment. Thus, we used uninformative beta(1,1) priors in the models for Se, Sp and prevalence for each latent status. The posterior distributions of the estimates of the default model were reported as medians with their corresponding 95% posterior probability interval (PPI). Sensitivity analyses were performed to check assumptions of LCA. Therefore conditional covariance was modeled between CHROMLAB and CHROMFARM, CHROMLAB and BC, MINLAB and MINFARM, and MINLAB and BC to check the assumption of conditional independence, and populations were excluded from the model to check whether Se and Sp are stable across populations (Toft et al., 2005).

Results and discussion

Milk samples from 154 cows were available for test evaluation. All were cultured with BC. Of these, 79 were cultured with CHROMagar both on-farm and in the laboratory, originating from 55 clinical mastitis cases and 24 subclinical mastitis cases. For Minnesota, 75 samples were cultured on-farm as well as in the laboratory, originating from 49 clinical mastitis cases and 26 subclinical mastitis cases.

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Table 1. Cross-tabulation of the number of quarter milk samples collected from cows with mastitis being positive (+) or negative (-) for 'no growth', Gram-positive growth and Gram-negative growth using bacteriological culture in the laboratory (BC, *n* = 154), CHROMagar or Minnesota on farm (FARM), and CHROMagar or Minnesota in the laboratory (LAB). Results are stratified to CHROMagar Mastitis[1] (*n* = 79) and Minnesota Tri-plate[2] (*n* = 75), with fractions presented for each of the lines

	BC+								**BC-**							
	FARM+ LAB+		**FARM+ LAB-**		**FARM- LAB+**		**FARM- LAB-**		**FARM+ LAB+**		**FARM+ LAB-**		**FARM- LAB+**		**FARM- LAB-**	
	n	**fraction**	*n*	**fraction**	*n*	**fraction**	*n*	**fraction**	*n*	**fraction**	*n*	**fraction**	*n*	**fraction**	*n*	**fraction**
CHROMagar																
'No growth'	6	0.08	2	0.03	4	0.05	5	0.06	12	0.15	8	0.10	7	0.09	35	0.44
Gram-positive	23	0.29	1	0.01	13	0.16	13	0.16	3	0.04	6	0.08	2	0.03	18	0.23
Gram-negative	5	0.06	3	0.04	1	0.01	3	0.04	1	0.01	9	0.11	1	0.01	56	0.71
Minnesota																
'No growth'	4	0.05	1	0.01	5	0.07	1	0.01	3	0.04	4	0.05	11	0.15	46	0.61
Gram-positive	40	0.53	6	0.08	4	0.05	3	0.04	1	0.01	7	0.09	2	0.03	12	0.16
Gram-negative	1	0.01	4	0.05	2	0.03	2	0.03	0	0.00	4	0.05	2	0.03	60	0.80

[1] CHROMagar Mastitis (CHROMagar, Paris, France)
[2] Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA)

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Table 2. Posterior median estimates of sensitivity (Se) and specificity (Sp) with corresponding 95% posterior probability interval (PPI) for CHROMagar[1] and Minnesota[2] used in the laboratory and on-farm and bacteriological culture using a Bayesian latent class model based on mastitis milk samples collected in 15 herds to detect 'no growth', Gram-positive bacteria or Gram-negative bacteria

		Bacteriological culture (*n* = 154)			**CHROMagar (*n* = 79)**						**Minnesota (*n* = 75)**					
					Laboratory use			**On-farm use**			**Laboratory use**			**On-farm use**		
		estimate	**95% PPI**		**estimate**	**95% PPI**		**estimate**	**95% PPI**		**estimate**	**95% PPI**		**estimate**	**95% PPI**	
			low	**high**		**low**	**high**		**low**	**high**		**low**	**high**		**low**	**high**
'No growth'	Se	0.40	0.25	0.58	0.71	0.51	0.91	0.63	0.44	0.84	0.64	0.33	0.96	0.38	0.18	0.65
	Sp	0.95	0.87	1.00	0.89	0.73	0.99	0.87	0.70	0.99	0.87	0.72	0.98	0.94	0.82	1.00
Gram-positive bacteria	Se	0.95	0.87	1.00	0.91	0.73	0.99	0.62	0.45	0.79	0.90	0.77	0.99	0.91	0.79	0.99
	Sp	0.70	0.56	0.85	0.86	0.69	0.98	0.78	0.62	0.90	0.85	0.64	0.99	0.62	0.41	0.81
Gram-negative bacteria	Se	0.46	0.25	0.73	0.34	0.15	0.62	0.59	0.32	0.86	0.23	0.07	0.50	0.37	0.15	0.69
	Sp	0.99	0.94	1.00	0.98	0.92	1.00	0.91	0.79	0.99	0.98	0.90	1.00	0.96	0.86	1.00

[1] CHROMagar Mastitis (CHROMagar, Paris, France)
[2] Minnesota Easy Culture System II Tri-plate (University of Minnesota, St Paul, MN, USA)

The cross-tabulated results of the evaluated tests are shown in Table 1. According to all tests, Gram-negative bacteria were least prevalent (prevalence ranging from 0.07 to 0.23), followed by 'no growth' (prevalence ranging from 0.16 to 0.37), and Gram-positive bacteria (prevalence ranging from 0.42 to 0.72).

Estimates of Se and Sp for the three latent statuses are given in Table 2. For Gram-negative and 'no growth', Sp was substantially higher than Se, but for Gram-positive, Se was higher than Sp for most tests. Se for Gram-positive bacteria was ≥ 0.90 for all tests, except for CHROMFARM, which had a posterior Se of 0.62, being significantly lower than Se of BC. CHROMLAB and MINLAB had similar test characteristics, but CHROMFARM and MINFARM differed substantially, particularly in Se. This suggests that although the two tests function similarly in the laboratory, farmers obtain substantially different results using the two tests.

The three latent statuses of bacteria in milk samples were used as a proxy for the IMI status of a cow, which enables farmers to target antimicrobial treatment. In this study we only determined whether bacteria were or were not present in the milk sample, but did not evaluate whether these bacteria originate from an IMI. Thus, we evaluated test characteristics with respect to finding bacteria in milk, irrespective of factors influencing that presence, such as shedding patterns of bacteria (Sears et al., 1990) or invasion of bacteria in mammary epithelial cells (Barkema et al., 2006).

Three assumptions need to be met if LCA is used: the compared tests are independent given disease status, the prevalence of the disease differs between populations, and Se and Sp of the tests is equal across different populations (Hui and Walter, 1980; Toft et al., 2005). Considering independency of tests, it is important to realize we are not evaluating the IMI status, as discussed above, but we evaluate different methods to measure whether culturable bacteria are present in the milk. If we would have evaluated the Se and Sp of the tests under comparison to detect an IMI, then all tests were likely dependent because all tests use a similar mechanism (i.e.: a culture-based protocol) to determine the IMI status. However, given the latent statuses defined above, we believe that the various culture-based tests we used can be seen as largely conditionally independent, because reasons for testing positive or negative, given the true latent status, likely differ between the tests. First, different types of milk samples were used: fresh milk samples were used for CHROMFARM and MINFARM and frozen and thawed milk samples for CHROMLAB, MINLAB, and BC. Freezing increases the

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probability to find Gram-positive bacteria from samples that were found to have no bacterial growth in fresh samples, and decreases the probability to find Gram-negative bacteria (Schukken et al., 1989). Additionally, BC milk samples were frozen, incubated, and cultured again if no bacterial growth was found, which increases the probability to recover bacteria from the milk (Sol et al., 2002). Second, the tests were performed at different locations, on farm and in the laboratory. Different locations imply, for example, different storage conditions which might affect chances of recovery of bacteria, but also different experience level of the readers, which likely affected the results. Third, different culture techniques were used, being a cotton swab for CHROMagar and Minnesota or an inoculation loop for BC. Cotton swabs retained more milk (approximately 0.15 mL) which results in a higher probability of finding bacteria from the milk plated (Wilkins et al., 1972). Fourth, the types of culture media differ. Blood agar used for BC is a nonselective medium, whereas CHROMagar consists of selective chromogenic media and Minnesota uses selective modified conventional media. Selective media might either support one type of growth, but suppress another type of bacterial growth, which likely affect the probabilities of pathogens to be recovered with the different tests. Nevertheless, some conditional dependence may still be present, specifically when comparing the same test in different circumstances (CHROMFARM vs CHROMLAB and MINFARM vs MINLAB) and between tests that were cultured with the same milk sample (CHROMLAB or MINLAB vs BC), as an example of observer related dependence (Menten et al., 2008). Therefore, we ran models assuming conditional covariance between CHROMFARM and CHROMLAB, between CHROMLAB and BC, between MINFARM and MINLAB, and between MINLAB and BC for each latent status and compared the results to the models assuming conditional independence, the default models. For the latent status 'no growth', modeling conditional covariance between the mentioned tests resulted in only minor changes in estimates compared to the default model. If changes were seen, the Se estimate differed with 11% or less. The Se estimate for MINLAB increased with 19% when allowing conditional covariance between MINFARM and MINLAB, indicating that MINLAB scored the false negative results of MINFARM often as negative too. Apparently, Minnesota reacts to something else than bacteria in the milk that is interpreted as bacterial growth (Wang et al., 2017). For the latent status Gram-positive bacteria, Se and Sp estimates differed with 11% or less as compared to the default model. Thus, the PPI we found likely includes the Se and Sp for the different tests to detect Gram-positive bacteria. For the latent status Gram-negative bacteria, modeling conditional covariance between the tests hardly affected Sp estimates, but Se estimates differed for all tests with 24% or less compared to the

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default model. As also reflected in the large PPI in the default model, we assume that the Se estimates mainly changed due to uncertainty given the low number of positive Gram-negative results instead of due to biased results.

The second assumption for LCA is about different disease prevalences between populations. The estimated true prevalences of the three latent statuses were found to differ at least 40% between the 30 populations, and thus were considered to differ substantially, allowing us to obtain reliable estimates (Toft et al., 2005). The third assumption for LCA regards Se and Sp of the tests being equal across different populations. A different farmer for each population may, however, have an effect on Se and Sp. Therefore, we checked whether Se and Sp were constant across the populations, and thus deleted different populations at each run, 1 by 1. In most models, Se and Sp estimates differed less than 9%, and for Gram-negative, Se estimates differed with 17% or less compared to the default model.

If tests are used to determine treatment of mastitis, it depends on the status of interest whether a high Se or a high Sp is preferred. Generally, tests performed in the laboratory seem to be preferred over the tests used on-farm. Of the tests used in the laboratory, BC has the highest Se with a small PPI for Gram-positive bacteria. This test would result in the lowest number of false negative results and might be favorable from the farmers' perspective. However, from a prudent antimicrobial use perspective, CHROMLAB should be selected based on the lower number of false positive results. If the tests are used by farmers to determine mastitis treatment against Gram-positive bacteria, MINFARM would be preferred to target treatment.

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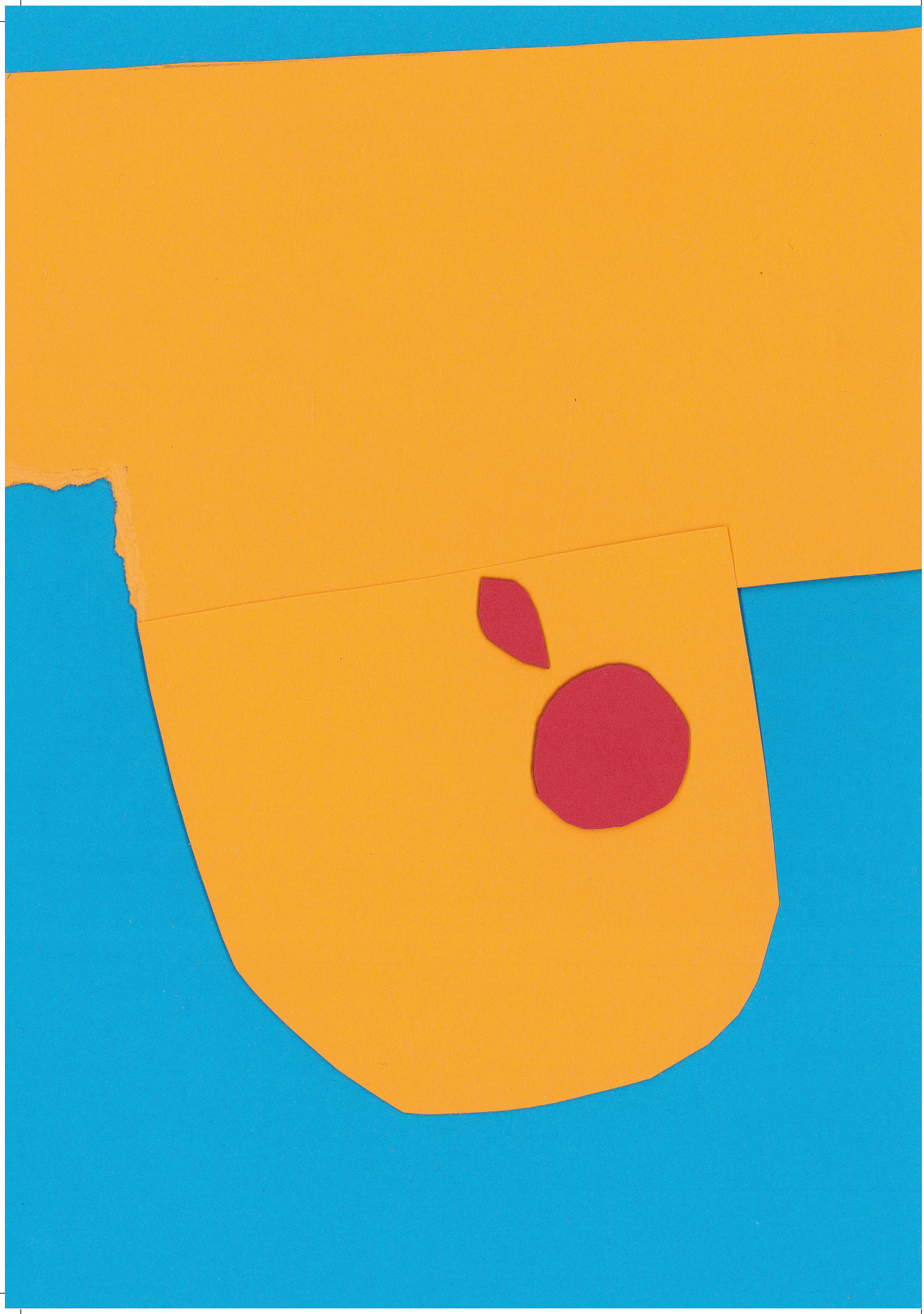

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8

General discussion

The potential role that antimicrobial usage on farms plays in the development of antimicrobial resistance in humans has raised an increased societal concern (Bager et al., 2000; Ruegg, 2018). In the Netherlands, this has led to regulations on antimicrobial use in livestock and the founding of the Taskforce Antibiotic Resistance in Animal Husbandry, both with the aim to reduce and optimize antimicrobial usage in livestock. Apart from the situation in humans, antimicrobial resistance in animals themselves is also important, because of the effect it may have on animal health and welfare through possible treatment failure. For both humans and animals, besides the impeding effect of reduced antimicrobial usage on the development of antimicrobial resistance, this process would also be delayed by prudent use of antimicrobials. In dairy cattle, treatment of mastitis, both at drying off and during lactation, is the most important reason to use antimicrobials. In the past decade much attention has been given to the reduction of antimicrobial usage at drying off (Scherpenzeel et al., 2014). Mastitis treatments during lactation received attention with respect to the length of treatment (Swinkels et al., 2013), and whether treatments should be applied at all (van den Borne et al., 2010). Optimizing mastitis treatment through the use of diagnostics, however, may be another way to optimize antimicrobial use in dairy cattle. Therefore, this thesis studied the added value of bacterial information on the mastitis treatment strategy of dairy farmers to contribute to improved mastitis treatments on Dutch dairy farms, and to, ultimately, enhance prudent antimicrobial use for mastitis treatments.

Current treatment strategy for mastitis

The taskforce on antimicrobial usage in cattle initiated different actions to ban preventive use of antimicrobials and to limit the use of critical important antimicrobials. To reach that goal, a herd health plan and a herd treatment plan were implemented on farms, among other actions (Speksnijder et al., 2015; Lam et al., 2017). The herd specific treatment plan is edited by the veterinarian, and lists all frequently occurring diseases with the preferred treatment for the specific herd. Also for mastitis, the preferred treatments are described, categorized according to the severity of mastitis. For clinical mastitis, three grades of mastitis are described, being grade one mastitis: abnormal milk; grade two: local signs on the udder, like redness or swelling; and grade three: systemic signs, like fever or anorexia. The rationale behind this categorization is that mastitis cases with different severity may have different causes and thus may need a different treatment. Generally, more severe cases are assumed to be caused by Gram-

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negative bacteria, whereas non-severe cases are more likely to be caused by Gram-positive bacteria. At the time the questionnaire described in *Chapter 2* was conducted, all farmers had to have a herd treatment plan, and by that, had a guideline to decide on treatment. In that questionnaire, farmers indicated to mainly use clinical signs in case of clinical mastitis to determine treatment. Nevertheless, 72% of the respondents indicated to have a need for additional tests to support treatment decisions, if such a test could be used on farm. Apparently, farmers need information in addition to the clinical signs to decide on mastitis treatments, but that information should be relatively easy available.

As opposed to the categorization in the herd specific treatment plan, it is known that the cause of mastitis cannot be predicted by the severity of signs (Ruegg, 2018), and that non-severe Gram-negative mastitis cases are also often found, and vice versa. An example of that is a Belgian prevalence study that found that up to 14.4% of the subclinical mastitis cases on farms was caused by coliforms (Piepers et al., 2007). Moreover, in our field study 22% of the clinical mastitis cases were found to be caused by Gram-negative bacteria (*Chapter 6*). Although a number of grade three mastitis cases were included, most of the enrolled cases were grade one or grade two mastitis. Furthermore, pathogen prevalence on farms has changed over the last decades from contagious pathogens such as *S. aureus* and *S. agalactiae* to more environmental bacteria such as coliforms and other Gram-positive bacteria (Sampimon et al., 2009). This shift in pathogen prevalence complicates the mastitis treatment decision strategy both during lactation and at drying off (Royster and Wagner, 2015), because this shift is from mainly Gram-positive bacteria to both Gram-positive and Gram-negative bacteria causing an IMI. These two categories need different treatment strategies.

Antimicrobial treatments for mastitis

As compared to the usage of 2009, antimicrobial usage in the dairy sector has been reduced by 47% (Figure 1), to a mean of 2.1 defined daily dosages for animals (DDDA) per year in 2018 (Geijlswijk et al., 2019). This reduction was the result of the regulations and great effort of the dairy sector.

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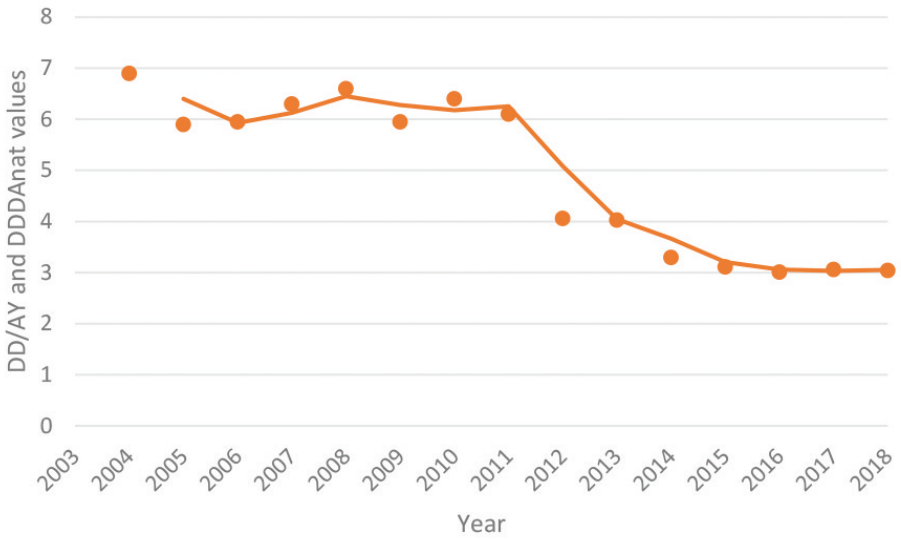

Figure 1. Long-term developments in antibiotic use according to LEI Wageningen UR data (in DD/AY, as published in MARAN reports, until 2010) and SDa data (in DDDAnat, from 2011 onwards), as point estimates for each year for the dairy cattle farming sector in the Netherlands, based on Geijlswijk et al. (2019)

Because antimicrobials should be kept available to treat diseased animals, reducing antimicrobial usage even further might not be preferred. This is agreed upon by the SDa, who has set the antimicrobial usage goals for the dairy cattle sector for 2019 equal to the goals of 2018, and deemed the current antimicrobial usage values as being acceptable (Geijlswijk et al., 2019). Despite the reduction, still most of the antimicrobials were used intramammary: for dry-cow treatment (1.1 DDDA) and mastitis treatments (0.6 DDDA) (Geijlswijk et al., 2019). Therefore, optimizing intramammary treatments remains an important step to enhance prudent antimicrobial treatments on dairy farms.

If an antimicrobial treatment is considered for mastitis, categorization of cows into different diagnostic categories enables targeted antimicrobial treatment. For that, three categories would suffice (Krömker and Leimbach, 2017), based on the group of pathogens found in milk samples: Gram-positive bacteria, Gram-negative bacteria, and no bacteria ('no growth'). In the Netherlands, an approved narrow spectrum intramammary antimicrobial effective against Gram-positive bacteria is on the market since 2016 to be used for mastitis treatment during lactation. However, no approved narrow spectrum intramammary antimicrobial effective against Gram-negative bacteria is available (Formularium, 2016). For the latter category, only broad

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spectrum intramammary antimicrobials are available, both for treatments during lactation and at drying off. Furthermore, debate exists whether Gram-negative IMI need intramammary antimicrobial treatment at all (Roberson, 2012, Royster and Wagner, 2015, Fuenzalida and Ruegg, 2019), based on the high reported self-cure of Gram-negative IMI (Ruegg, 2018). However, in the Netherlands it is common practice to treat Gram-negative IMI with intramammary antimicrobials, and studies are lacking that prove the ineffectiveness of an antimicrobial treatment against Gram-negative bacteria under Dutch circumstances. Regarding the category 'no growth', no intramammary antimicrobial treatment is necessary, because no viable bacteria are detected and thus applying antimicrobials would not be prudent. Whether that is fully true can be debated because culture negative results might be caused by, for example, *S. aureus*, which can invade somatic cells (Barkema et al., 2006), and knows a cyclical shedding pattern (Sears et al., 1990), and thus may not grow in culture. Also the number of bacteria in the milk might be below the detection limit of the test applied. Therefore, when obtaining a 'no growth' result, it is never known whether the mammary gland is truly free of bacteria.

Diagnostic tools that provide information on the three diagnostic categories Gram-positive, Gram-negative, or 'no growth' may be useful for treatment differentiation. Because farmers consider proper sample collection hard and if they send in milk samples, samples are often found to be contaminated (*Chapter 5*), it has been studied whether such categorization is possible without the need of collecting milk samples. On dairy farms, much data is available, and if that data can be used to decide on mastitis treatment, that would be a convenient approach. In an US study, for example, an approach using farm management data combined with test-day record data was used to categorize cows at dry off into one of two groups: a group that might benefit from dry-cow antimicrobials (high risk) and a group that likely will not benefit from such a treatment (low risk) (Vasquez et al., 2018). For this categorization CSCC on the last three test-day records, and clinical mastitis history was used. The cows indicated as low risk cows could be dried off without antimicrobials, without differences in probabilities for new infections or for clinical mastitis up to the first 30 days of the next lactation. Although this approach helps to categorize cows as candidates for antimicrobial treatment or not, the type of treatment could still not be determined. Another study has investigated whether data collected in an automatic milking system is of use to predict the Gram-status of the bacteria causing clinical mastitis. The data used was electrical conductivity, color sensors blue, red and green, and milk yield. Due to the limited predictive value of the included variables, the Gram-status of the involved

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mastitis pathogens could not be determined (Kamphuis et al., 2011). Furthermore, not all farms have automatic milking systems. As most farms have CSCC data available, it has been studied whether clinical signs combined with SCC give enough information on the cause of the IMI to decide on mastitis treatment (de Haas et al., 2002; Jashari et al., 2016; Petzer et al., 2017). Although these studies conclude that different pathogens gave different SCC patterns, the patterns of the different pathogens showed too much overlap to differentiate between treatments. Thus, up to now no variables have been detected that have enough predictive value to be used for treatment differentiation. Therefore, direct information on the bacteria involved is still needed to differentiate among treatments for mastitis.

Farmers considered pathogen information important too, as they indicated to need additional information on the pathogen involved, or on the type of treatment to apply (*Chapter 2*). The type of antimicrobial should be chosen based on the Gram-status of the bacteria causing the mastitis. The decision whether or not a cow should be treated at all, should be based on the probability that a cow will recover. For that, cow factors have predictive value. Up to now, however, using cow factors to predict cure probabilities of cows and thus to select treatable candidates is considered to be highly underused in the mastitis treatment strategy (Barkema et al., 2006).

Intramammary infection and cure

The immune system of the cow forms the first line of defense when a pathogen invades. Many aspects influence whether the cow is able to clear the infection itself, and thus whether the cow might need an antimicrobial treatment or not. The ability of the cow to form a rapid and effective immune response depends on cow factors such as parity or days in milk, but also on the invading pathogen (Zadoks et al., 2001; Green et al., 2004; Ballou, 2012; Royster and Wagner, 2015). If an effective immune response is developed, the cow is able to self-cure the infection. The CSCC before infection influences the speed at which an immunological response is mounted and thus the severity and chronicity of mastitis (Bradley, 2002; Barkema et al., 2006), with lower CSCC levels resulting in more severe mastitis (Suriyasathaporn et al., 2000). However, not only the CSCC level at infection, but also nutritional status and a negative energy balance are reported to influence the immunological response (Bradley, 2002). Other factors that affect the cure probability are, among others, whether hind or front quarters are infected, with

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hind quarters having lower cure probabilities (Barkema et al., 2006), the number of quarters per cow infected (Barkema et al., 2006), and whether the cow experienced clinical mastitis earlier (Pinzón-Sánchez and Ruegg, 2011).

The invading pathogens, on the other hand, have evolved factors to survive the immunological response and to chronically infect the mammary gland. For *S. uberis* it has been described that it can resist phagocytosis and intracellular killing by leucocytes (Bradley, 2002). For *E. coli* and *S. aureus* it has been described that they are able to survive within neutrophils. Both species have also the capability to adhere to mammary epithelial cells (Bradley, 2002; Barkema et al., 2006). This latter survival mechanism has been described for *S. dysgalactiae* too (Bradley, 2002). For *S. aureus*, many additional aspects of surviving the immunological response have been described, like the ability to form small-colony variants, to induce fibrosis, and to form micro-abscesses (Barkema et al., 2006). In addition to that, the epidemiology of the IMI also depends on the site within the udder affected by the bacteria. Of streptococci, for example, it is known that they remain in the milk compartment, whereas *S. aureus* penetrates the udder tissue (Pyörälä, 2009).

Because both cow factors and the pathogen affect cure probabilities of cows, considerably different cure rates are reported for cows infected with the same pathogen. For *S. aureus*, for example, cure rates varying from 4% to 92% have been reported (Barkema et al., 2006), which might be caused by the existence of various strains of *S. aureus* with different pathological effects, or by a difference in the cows under study. All aspects together determine the probability that a cow cures from mastitis.

Point-of-care tests

Farmers have indicated a need for POC tests as part of their mastitis treatment strategy (*Chapter 2*). As mentioned in *Chapter 1*, POC tests gained increased attention over the last decade in veterinary medicine, which is reflected in the number of studies performed evaluating the use of such tests. In Figure 2 the number of studies (n = 928) reporting on POC tests for cattle are shown based on a search on SCOPUS with the query 'TITLE-ABS-KEY ((on-farm OR on-site OR cow-side OR point-of-care) AND (bovine OR cattle OR dairy) AND (test OR culture)). These tests range, for instance, from tests

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to diagnose hyperketonemia on dairy farms (Tatone et al., 2016), to tests to diagnose hypoglycemia (Zakian et al., 2017), to diagnose Infectious Bovine Rhinotracheitis virus (Hou et al., 2017), or Brucellosis in semen in resource-limited regions (Yang et al., 2018).

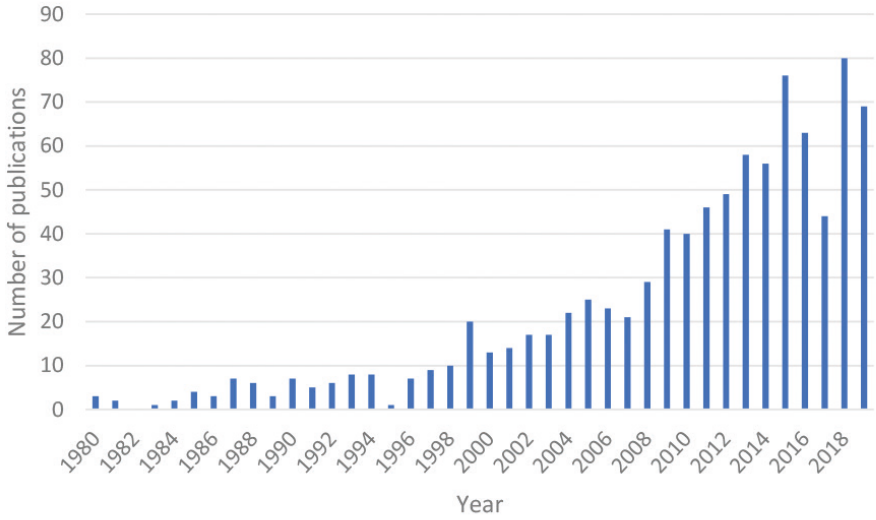

Figure 2. Number of publications per year from Scopus indicating the increase in research in bovine POC testing

Furthermore, on-farm tests have been evaluated that could, for example, detect uterine *E. coli* infections (Dubuc, 2017), or guide treatment for purulent vaginal discharge (Madoz et al., 2017). These two studies evaluated culture-based on-farm tests that also have been evaluated to detect the pathogen involved in an IMI. A selection of tests that can be used to detect the involved pathogen is shown in Table 1. Some of the listed tests are recently developed, and are evaluated during the time the research described in this thesis was conducted. All tests aim to lower the time-to-result and to support treatment decisions for mastitis.

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Table 1. Overview of POC tests and their characteristics to detect mastitis-causing pathogens to be used on dairy farms in the mastitis treatment strategy

Test	Bacteria	Accuracy
Accumast	Bacteria associated with clinical mastitis	Se 0.82, Sp 0.90
(Ganda et al., 2016)	*Staphylococcus* spp.	Se 0.70, Sp 0.95
	Gram-negative bacteria	Se 0.82, Sp 0.99
Mastatest	All targets	Se 0.95, Sp 0.72
(Jones et al., 2019)	*Streptococcus uberis*	Se 0.88, Sp 0.80
	Coliform bacteria	Se 0.77, Sp 1.00
mastDecide	Gram-positive cocci	Se 0.84, Sp 0.94
(Leimbach and Krömker, 2018)	Coliform bacteria	Se 0.72, Sp 0.83
	No growth or further pathogens	Se 0.71, Sp 0.91
Minnesota Easy Culture S-II Bi-plate	Gram-positive bacteria	Se 0.83, Sp 0.83 (average)
(Royster et al., 2014)	Gram-negative bacteria	Se 0.65, Sp 0.95 (average)
	No growth	Se 0.78, Sp 0.89 (average)
Minnesota Easy Culture S-II Tri-plate	Gram-positive bacteria	Se 0.83, Sp 0.85 (average)
(Royster et al., 2014)	Gram-negative bacteria	Se 0.69, Sp 0.97 (average)
	No growth	Se 0.80, Sp 0.88 (average)
Overnighter and Staphalert	*Streptococcus* spp., *Staphylococcus* spp.	No literature available
Petrifilm	Gram-positive bacteria	Se 0.90, Sp 0.88
(AerobicCount&ColiformCount)	Gram-negative bacteria	Se 0.85, Sp 0.75
(Mansion-de Vries et al., 2014)	No growth	Se 0.41, Sp 0.91
Speed Mam Color	Various bacteria	No literature available
VétoRapid	Gram-positive bacteria	Se 0.91, Sp 0.78
(Viora et al., 2014)	*Escherichia coli*	Se 0.58, Sp 0.98

Se = sensitivity, Sp = specificity

Genotypic versus phenotypic tests

Farmers preferred a time to result of no more than 8 h for clinical mastitis. Because all tests listed in Table 1 use some form of culture, they can provide a result within 24 h (Ganda et al., 2016; Leimbach and Krömker, 2018; Jones et al., 2019). Consequently, none of the tests fulfill farmers' preferences in that perspective. If culturing would not be necessary, time would be saved. Based on screening possible platforms for development of new tests, LAMP was selected in this thesis as a platform that might be suitable for on-site use (outside a central laboratory). Although literature reports on the use of LAMP for mastitis pathogens (Appelt et al., 2019), and on the on-site use of

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LAMP (Tian et al., 2018), no study on an on-site LAMP for mastitis-causing pathogens was available. As described in *Chapter 4*, LAMP was found to generally have a high agreement with BC. However, due to the time and skills needed for DNA extraction, the part concerning sample preparation is not yet suitable for on-site use. More simple sample preparation protocols have been described for LAMP assays (Lee et al., 2019), but no such protocol is available yet to be used on milk samples. Important is that a simplified sample preparation protocol does not affect the performance of the LAMP assays. Disregarding the DNA extraction, however, the runtime was short. Within one hour all LAMP assays could show a result, which fit the requirements of the farmers and thus give perspective to fast on-site testing of milk samples.

A result of a genotypic test, like PCR or LAMP, might be of different meaning than a result of a phenotypic test, like BC. Genotypic tests reproduce DNA present in the milk, regardless if DNA is from viable or non-viable bacteria. Phenotypic tests, on the other hand, only might become positive if viable bacteria are present, because bacteria need to grow on the culture medium before they can be observed. In recent infections, both types of tests would probably result in the same outcome as up to 36 h after challenge, agreement between BC and PCR was reported to be high (to detect *Staphylococcus spp.*: kappa 0.85). At a later stage, agreement dropped to moderate (kappa 0.58) due to the low number of colony forming units present in the milk (Hiitiö et al., 2018). This low number of viable bacteria in the milk might be below the detection limit of BC. The number of viable bacteria can be reduced by an effective immune response that kills most bacteria. An additional consideration for LAMP is the Tp cutoff set, which affected the determined Se and Sp as shown in *Chapter 4*. For PCR it has been reported that a shift of the cycle threshold (Ct) affects the definition of disease status under study (Cederlöf et al., 2012), with a higher Ct threshold indicating a lower number of viable bacteria in the milk. Whether the change in Se and Sp at different Tp cutoffs was caused by a different bacterial load in the milk samples was not determined, but might be interesting for further research. Furthermore, genotypic tests are limited to detect the targets for which the used primers are designed. In contrast, BC has a wider scope, although that test too has limits, such as specific growth conditions for bacteria like *Mycoplasma* spp. Such limits also affect the performance of on-farm tests that often use selective media, where one medium enhances growth of specific (groups of) bacteria, but suppresses other growth.

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Test performance

All tests mentioned in Table 1 are able to detect various mastitis-causing pathogens. The evaluation of four culture-based tests in the laboratory on 866 milk samples revealed that specification to species level is often less reliable than to categorize the result into one of the three diagnostic categories (*Chapter 3*). This was supported by the laboratory evaluation of the Minnesota Bi- and Tri-plate (Royster et al., 2014). As the aim of the thesis is to determine whether such tests can contribute to an improved mastitis strategy, and the three diagnostic categories should be sufficient for treatment differentiation, the advice would be to not use these tests for further specification of bacteria.

For that treatment differentiation, none of the tests investigated in *Chapter 3* was favored as each test showed similar agreement with BC. Most of the tests listed in Table 1 are capable to detect the three diagnostic categories that were indicated in this thesis as being at least necessary to determine treatment (Gram-positive bacteria, Gram-negative bacteria, and 'no growth'). However, it is important to be aware of the variation that exists between the tests regarding the pathogens that are considered within these three categories. In addition to that, although most studies used milk samples of cows with clinical mastitis, there is evidence that Se and Sp might vary due to prevalence differences (Brenner and Gefeller, 1997; Li and Fine, 2011), and thus the obtained Se and Sp in those studies probably not generally apply if these tests are used on dairy farms. Moreover, most studies used BC as the reference test, of which is known that is has limited Se and Sp. Therefore, the misclassification made by BC affected the determined Se and Sp of the evaluated tests. Furthermore, as shown in *Chapter 7*, test characteristics are not only affected by the test itself, also the interpreter or sample storage, for example, influences the ability of the tests to detect mastitis-causing pathogens.

All these aspects together determine whether a test is able to detect mastitis-causing pathogens. Because intramammary treatments are directed against mastitis-causing pathogens infecting the udder cistern, the ultimate goal for treatment direction would be a test indicating whether mastitis-causing bacteria are present in the udder, and which bacteria. However, up to now, the best there is are tests that detect bacteria present in the milk. Therefore, the effect of sample collection on the recovery of bacteria has been studied with real-time PCR (Hiitiö et al., 2016). For that, milk was

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collected immediately from the udder cistern with a needle and vacuum tube, and was compared to milk that was collected conventionally from cows with CSCC ≥ 200,000 cells/mL and a CMT positive quarter. They found more samples without bacteria and more samples with one bacterium when the milk was collected immediately from the udder cistern as compared to conventional sample collection. Furthermore, more than twice as much *Staphylococcus* spp. were found with conventional sample collection, compared to the samples collected immediately from the udder. The bacteria in the conventionally collected samples originated from outside the udder cistern, for example, from the teat canal, or from the environment, due to contamination at sample collection. The effect of presampling procedures, such as cleaning the teats, removing the first streams of milk, and disinfecting teats with 70% alcohol, was described. They found that proper presampling procedures reduced the odds ratio for PCR-positive samples to 0.75 as compared to not applying such procedures when collecting milk samples (Mahmmod et al., 2013a). Given the aspects on sample collection and test performance, none of the available tests can predict the real IMI status. Therefore, it is of utmost importance to apply correct sample collection, to minimize the effect of contamination on test results and to obtain the truest reflection of the intramammary status, given the test potentials. Proper sample collection is even more important if on-site tests are applied: in a laboratory, a trained laboratory technician interprets the result of the test. In the case of BC most often slight contamination is ignored, or contamination is reported if the sample is more heavily contaminated. In both cases, the technician is trained to judge the result and to determine whether the result of BC likely is relevant to be used to decide on treatment. If a farmer would use an on-farm test, this judgment lacks without proper training and experience. It might be that farmers would interpret contaminated results as growth and would treat such cases accordingly, although treatment might not be effective.

In a central laboratory regular quality checks are performed to guarantee the reliability of the results. As discussed, various aspects influence the test potential to inform on the IMI status and thus whether the test is of value to be incorporated in the treatment strategy of farmers. Although numerous studies determined Se and Sp of tests and concluded that these tests can be used on-farm, only limited number of studies actually determined whether incorporating on-farm testing in the treatment strategy improved treatment of mastitis. Moreover, not only the test itself influence the performance, also the performer, e.g. the farmer, affects the test performance. Therefore, tests should be validated at the site of intended use (Abuelo and Alves-Nores, 2016; Leimbach and

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Krömker, 2018), by the person who is intending to use the tests. The performer effect is incorporated in the latent class analysis as described in *Chapter 7*, and is one of the aspects why the tests were assumed to be conditionally independent. Because the performer has an effect on the performance of the test, proper training, providing protocols, and gaining experience are important to make sure tests are interpreted and used correctly. Additionally, like in the central laboratory, regular checkups on the execution and interpretation of the test is crucial to guaranty quality of the results. For that, a system might be set-up where known samples are evaluated. Such an approach is considered of critical importance to determine the analytical performance of the POC tests on-farm. Only then, the validity of the tests can be judged and guaranteed.

Farmers indicated to prefer less than 7% false results (*Chapter 2*). As farmers will work with the tests, this number means that of the results obtained by the farmers, 7% is accepted to disagree with the true status of the cow. Thus, that number is referring to a predictive value rather than Se and Sp. Estimates of Se and Sp are hard to give, because predictive values depend on prevalence of the disease in the population (Brenner and Gefeller, 1997). Moreover, it is even more complicated due to that the false results are based on the bacteria in the milk that are detected or not detected by the test. For treatment decisions, however, information on the presence or absence of mastitis-causing bacteria in the udder is of interest. For the misclassification likely present between the bacteria in the milk and the actual mastitis-causing bacteria in the udder, however, cannot be corrected as too many aspects have an effect on that to give reliable estimates. Further, whether a high Se or a high Sp is preferred depends on the purpose of the test. The tests as used in this thesis (*Chapter 5* and *6*) were tests used for treatment differentiation. As such, Se and Sp preferences depends on the diagnostic category to be detected. For the 'no growth' category, a high Sp likely is preferred. A high Sp implies a low number of false positive results. For 'no growth' that means that a low number of cases is diagnosed as 'no growth', whereas they actually are infected. And thus most of the infected cows will have a negative result for 'no growth'. All evaluated tests were found to have a high Sp for 'no growth' in *Chapter 7*. For the Gram-positive category, however, a high Se would be preferred. In that case the least number of milk samples with Gram-positive bacteria would be missed and therefore, most infected cows would receive an antimicrobial treatment. Farmers too, have indicated to prefer a higher number of false positive results over a high number of false negative results at drying off in *Chapter 5*. At drying off, this is as expected because farmers do not have to discard milk and therefore apply a 'better

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safe than sorry' approach. However, whether that is also the case for a test applied during lactation could be discussed. For example, in case of mild clinical mastitis a false negative result might be not too bad. If no antimicrobial treatment would be applied in such a case, and if such a case would not cure within a few days, the farmer may still start an antimicrobial treatment. But all variations apply and thus tests should be evaluated at the site of use to determine whether they are of added value.

Mastitis treatment strategy and diagnostic information

If POC tests for mastitis are incorporated in the treatment decision-making process, they should provide valuable information to support that decision. The performance of the tests might be of less importance (Abuelo and Alves-Nores, 2016; Busin et al., 2016), as long as it meets acceptable performance in comparison to laboratory testing and related to the clinical outcome (St-Louis, 2000). Because in case of mastitis, on-farm testing is not a replacement of another test, but an add-on test to the treatment decision strategy, the effect of using such test on the decision-making process and specifically on the outcome, is of greatest interest over the performance as compared to a laboratory test.

Often is assumed that POC tests are performing less that laboratory tests (Busin et al., 2016). In *Chapter 7* it was found that BC often had similar Se and Sp estimates as had the other tests. However, if farmers and veterinarians have the idea that such tests are of inferior quality, they might be less motivated to incorporate such tests in the treatment strategies for mastitis. Adding bacterial information to the mastitis treatment strategy resulted in more targeted treatments than without testing (*Chapters 5* and *6*), and thus, a treatment strategy using such tests might be advocated in light of prudent antimicrobial use. Moreover, as antimicrobials only are effective against viable bacteria, the presence of bacteria should be determined before applying an antimicrobial treatment. If prudent antimicrobial use for mastitis would be requested, incorporating additional testing into the mastitis treatment strategy might be an approach. A few strategies may contribute to change the current treatment strategy of farmers. One of these is by convincing veterinarians of the necessity of using on-farm tests to improve mastitis treatments. Veterinarians are found to have an influencing role on treatment duration of farmers (Swinkels et al., 2015), and on the opinion of farmers regarding prudent antimicrobial use (Vasquez et al., 2019), and thus they might have an effect on the type of treatment

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farmers choose too. Farmers are also sensitive to ideas of other farmers (Vasquez et al., 2019), and if colleagues consider using on-farm tests good stockman ship, they will be more likely to incorporate tests too in their treatment strategy. If farmers perceive being capable to use antimicrobials prudently, they were found to have a positive intention to doing so (Vasquez et al., 2019). Thus, providing tests to the farmers for on-farm testing might give farmers the confidence of being capable to use antimicrobials prudently. As social pressure was found to be one of the aspects affecting the likeliness of changing behavior with respect to reducing antimicrobial usage at drying off (Lam et al., 2017), that might also be of help to incorporate additional testing in the treatment strategy for mastitis during lactation. Eventually, to change the mastitis treatment strategy on Dutch dairy farms nationwide, all aspects of the RESET model need to be considered (Lam et al., 2017), and will be discussed later.

During the time this research was conducted, some important aspects affected the dairy industry in the Netherlands. In 2013, legislation on the restrictive use of antimicrobials was implemented. In 2015, milk quota disappeared and anticipating on that, less cows were culled in the years before. In 2016, narrow spectrum intramammary antimicrobials were introduced against Gram-positive bacteria. Until then only broad spectrum intramammary antimicrobials were available for lactational mastitis treatments. In 2017, all farms had to reduce their phosphate production by, on most farms, lowering the number of cattle. In 2018, antimicrobial usage was almost reduced by half compared to 2009. All these aspects likely had affected farm management. For example, due to the limited head of cattle that farmers were allowed to have on their farms, farmers lowered the number of youngstock. Consequently, replacement likely is less easy and longevity of cows becomes more important. Furthermore, also in terms of sustainability, farmers are encouraged to increase the longevity of cows on their farm.

The need for additional information for mastitis treatment strategies during lactation that farmers indicated in 2014 (*Chapter 2*), was still present among the farmers in 2018 (*Chapter 6*). This was expected given the changes in the dairy industry and the increased importance of treating mastitis in the best possible way. At drying off, however, the need for additional information was only limited. Probably, farmers considered SCC substituted with information on mastitis history and milk yield enough to make an informed treatment decision (*Chapter 5*). This was already indicated in 2014, when they just recently had adopted selective dry-cow treatment based on, most often, CSCC data. Repeating this question two years later and actually providing bacterial information to

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the farmers, did not change their need. At drying off the decision is mainly to treat or not to treat with an antimicrobial as only one type of antimicrobial (cloxacillin) is approved in the Netherlands for this indication. However, the spectrum of this antimicrobial is against Gram-positive bacteria and thus Gram-negative bacteria are not treated at dry off. If Gram-negative bacteria are involved with the mastitis at drying off, other dry-cow antimicrobials need to be chosen. These antimicrobials are broad spectrum antimicrobials, and categorized as second choice. These antimicrobials can only be used if their need is supported by herd history on mastitis caused by Gram-negative bacteria, and only for a limited time. Even though farmers indicated the lowest need for diagnostics at drying off (*Chapter 2*), working with bacterial information improved the treatment decisions with respect to the bacterial cause of the mastitis (*Chapter 5*). Nevertheless, the extra work and costs were not considered to outweigh the added value of this type of information. As Gram-positive pathogens likely form the largest group of pathogens at drying off, cloxacillin products are found to be equally effective as other dry-cow antimicrobials (Halasa et al., 2009). With that in mind, the low need of farmers for additional information is not surprising. Moreover, legislation on restrictive antimicrobial use and the ban on preventive use of antimicrobials, had not affected udder health negatively on Dutch dairy farms (Santman-Berends et al., 2016; Vanhoudt et al., 2018), and likely farmers consider the current approach at drying off effective. Another study that incorporated bacterial information in the dry-cow treatment strategy of farmers concluded that antimicrobial usage was lower if such information was included than if not (Cameron et al., 2014). However, they compared the effects to a control group where blanket dry-cow treatment was applied and used low SCC cows. In the Netherlands, farmers are already used to applying selective dry-cow treatment, and thus our results deviated from the referred study. Nevertheless, as concluded in *Chapter 5*, use of diagnostic information in high CSCC cows at drying off would be the optimal approach in terms of antimicrobial usage, cost and time saving, with the same number of incorrect dry-cow treatment decisions. But, because more false negative results were obtained in this approach as compared to the current treatment strategy at drying off, the effects on udder health parameters during the dry period and in the next lactation should be studied.

For mastitis treatments during lactation, farmers probably include cow factors in their current treatment strategy, according to the cure results described in *Chapter 6*. Nevertheless, they question their treatment decisions and thus consider the currently available information not specific enough to differentiate between treatments. However,

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based on the results for subclinical mastitis, no beneficial effect was observed in terms of increased antimicrobial usage without improving bacteriological cure. Nevertheless, farmers are willing to incorporate these tests in their subclinical mastitis treatment strategy. As reflected in the third level of decision making of mastitis treatments (Vaarst et al., 2002), for cows with subclinical mastitis cow factors are important to be regarded before an antimicrobial treatment is considered. Given the results of this thesis, on-farm testing is not generally advised for subclinical mastitis, but may be considered for recently infected cows. Recently infected cows were found to have higher cure probabilities if treated (van den Borne et al., 2010), and thus early treatment of such cows may contribute to an increased longevity of cows. In case of clinical mastitis farmers often feel the urge to treat a cow immediately. The need to do something is a driver for antimicrobial use (Aarestrup, 2015). Adding on-farm tests, and thereby let farmers do something, e.g. collecting a milk sample and performing a test, might help farmers to postpone treatment and to decrease antimicrobial usage by giving the cows the possibility to self-cure. After working with those tests, most farmers participating in the field study were willing to continue the use, even though clinical cure was the same between the groups. Apparently, the added information provided by the tests outweighed the added time and money spent, even though cure was not affected positively. This might be caused by farmers striving for being a good farmer and to practice good stockman ship (Swinkels et al., 2015). Because farmers often question themselves when deciding on treatment, a test showing bacterial growth justifies the of use antimicrobials and by that, likely reduces this insecurity. Furthermore, farmers likely perceive culturing of milk samples for treatment decisions good farmer practice, which may be the result of years of advice of veterinarians. Education is one of the approaches to change behavior, forming the first E in RESET. Up to now, farmers could have thought or even experienced that incorporating culture results in their treatment strategies was not possible due to the delay between sampling and result. By bringing tests to the farm, this gap is bridged.

Generally, farmers have a positive mindset regarding prudent antimicrobial usage. Whether farmers actually will use antimicrobials prudently, depends on factors such as whether the strategy fits daily routine, and knowing what needs to be improved (Vasquez et al., 2019). However, other aspects must be visible to the farmers too like saving work and money and a positive effect on animal health. These latter two aspects were not met by the current method of incorporating tests in the treatment strategy for mastitis. Specifically for subclinical mastitis, where on-farm testing resulted in an

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increased antimicrobial usage, without a positive effect on bacteriological cure. Also for clinical mastitis, the current way of adding tests to the treatment strategy was found to be not cost-effective on most farms (Down et al., 2017). Because economics form the second E in RESET, this will not contribute to adopting on-farm tests in the treatment strategy for mastitis. Major components contributing to the fact that these tests were not found to be cost-effective, were the bacteriological cure probability and the proportion of Gram-positive cases. This cost estimation study considered bacteriological cure not to be improved by applying on-farm testing, in agreement with other studies (Lago et al., 2011a; Schmenger et al., 2018). In our study too, bacteriological cure probability of cows was also found to be comparable or less as compared to the current treatment strategy. From that perspective, using on-farm tests likely is not cost-effective on Dutch farms too. In addition, Down et al. (2017) found that if 20% or more of the clinical mastitis cases were caused by Gram-positive bacteria, an on-farm test approach would not be cost-effective. On most Dutch dairy farms the proportion of Gram-positive bacteria will be above 20%, and thus again, using on-farm tests likely will not be cost-effective. However, the cost estimates were based on a strategy were mastitis with Gram-negative results were not treated. In the Netherlands, these cases likely will be treated. Therefore, given the different approach of Gram-negative test results, the economic aspects of using on-farm tests should be determined under Dutch circumstances to give reliable estimates on the cost-effectiveness. Other important outcomes of treatment are whether the affected quarter and affected cow is saved and the speed to clinical recovery (Roberson, 2012). These aspects were not found to be deteriorated by applying on-farm tests as compared to the current treatment strategy (*Chapter 6*).

Final thoughts and conclusion

Adding bacterial information to the treatment strategy of farmers improved mastitis decisions on dairy farms because more treatments were targeted the bacteria present in the milk than if such information was not used. If the goal is to enhance prudent antimicrobial usage on Dutch dairy farms, incorporating POC tests in the treatment strategy for mastitis may be considered. To implement such tests in the field, the farmers' mindset should be changed with respect to the treatment strategy for mastitis. A strategy to do so is by providing all aspects of the RESET mindset model (Lam et al., 2017). Up to now, however, not all aspects of the RESET mindset model are met

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yet, as will be briefly discussed now. First, further Rules on restrictive antimicrobial use, the R in RESET, are not to be expected, given the acceptable low antimicrobial usage on dairy farms already. The second, Education, is an aspect that has been met already, because years of education probably led to the need that farmers indicated for additional information on the bacterium involved. However, the potential benefits of on-farm testing for individual farms has not been discussed in the Netherlands and only limited knowledge is currently available on that point. Third, Social pressure might have a supportive effect on the adoption of such tests, specifically if farmers are enthusiastic on the use of the tests and success stories will be told. Up to now, because such tests are hardly used, these stories are lacking in the Netherlands, but have been described in the US (Roberson, 2012). Fourth, an approach of on-farm testing likely is not advisable Economically given the estimated costs. However, the cost-effectiveness should be determined under Dutch circumstances too. Fifth, if adoption of these tests is requested, Tools to do so should be widely available, forming the T in RESET. Currently, on-farm tests are scarcely, but increasingly available to be used by Dutch dairy farmers for mastitis treatments. Other aspects that contribute to the technical facilities are regular checkups and for example a herd treatment protocol incorporating bacterial information for treatment differentiation. In addition to the RESET mindset model which may contribute to whether on-farm tests will be incorporated in the treatment strategy for mastitis, there are a number of reasons why providing such tests to the farmers would probably not result in enhanced prudent antimicrobial use for mastitis immediately.

At drying off, antimicrobial usage increased compared to the current treatment strategy when bacterial information was incorporated in the treatment strategy. Therefore, selective use of bacterial information might be an approach to further reduce antimicrobial usage, without effect on the number of incorrect decisions. Whether that information should be obtained from on-farm testing or laboratory testing probably depends on the preferences of the farmer and the veterinarian. However, in this thesis, the use of on-farm tests was not evaluated at drying off and the effect on udder health parameters has not been determined. Thus, further research is necessary to determine these effects, and whether on-farm testing might be in favor over laboratory testing at drying off.

For clinical mastitis farmers have a need for additional information to determine a treatment strategy. However, the strategy they currently apply, without additional

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information on the causative bacteria, results in equal or better cure probabilities for cows with clinical mastitis, than if such information is incorporated. Apparently, their treatment strategy is a complicated path in which multiple aspects are considered, based on experience, and cow factors such as days in milk, milk production, mastitis history, and severity of the mastitis. All these aspects cannot be captured in bacterial information alone. Nevertheless, the main interest of farmers is the pathogen involved and how to treat the cow, which can both be determined based on bacterial information provided by on-farm tests, although most often no antimicrobial susceptibility is available from such tests. Further research is necessary to determine which aspects need to be included in addition to bacterial information, to enhance prudent antimicrobials use with an improved cure. Particularly, because for antimicrobial use applies: as little as needed, as much a necessary. Part of that can be obtained from bacterial information, but future studies should reveal the information that needs to be included as well.

Furthermore, not only the urge will increase to use antimicrobials as little as possible and as much as needed. Also new diagnostics will be developed or those parameters in the available data with predictive value will be found. Eventually, diagnostics and cow information should be combined to provide farmers the additional information needed to enhance prudent antimicrobial use for mastitis. Examples of such decision trees are reported (Pinzón-Sánchez et al., 2011; Schmenger et al., 2018). However, until practical tests are available, the adoption of such an approach may be limited as then at least one of the cues to action is missing, the T of tools in the RESET mindset model. Nevertheless, setting up such a decision tree for Dutch dairy farms would be a step forward to enhance prudent antimicrobial use.

In conclusion, farmers indicated to have a need for bacterial information to incorporate in their treatment strategy for mastitis. Bacterial information is important to be used when deciding on mastitis treatment, to delay the process of antimicrobial resistance development. Only adding bacterial information to the treatment strategy for mastitis did not improve cure of mastitis as compared to the current treatment strategy farmers apply. Therefore, further research should focus on other aspects that apparently have a major impact on the cure probabilities of cows with mastitis, such as days in milk, mastitis history, or milk yield. These factors likely are, maybe unknowingly, incorporated by the farmers when deciding on mastitis treatment currently. Because these factors may be considerably important, information on both the pathogen and the cow should be combined, to enhance prudent antimicrobial use for mastitis on Dutch dairy farms.

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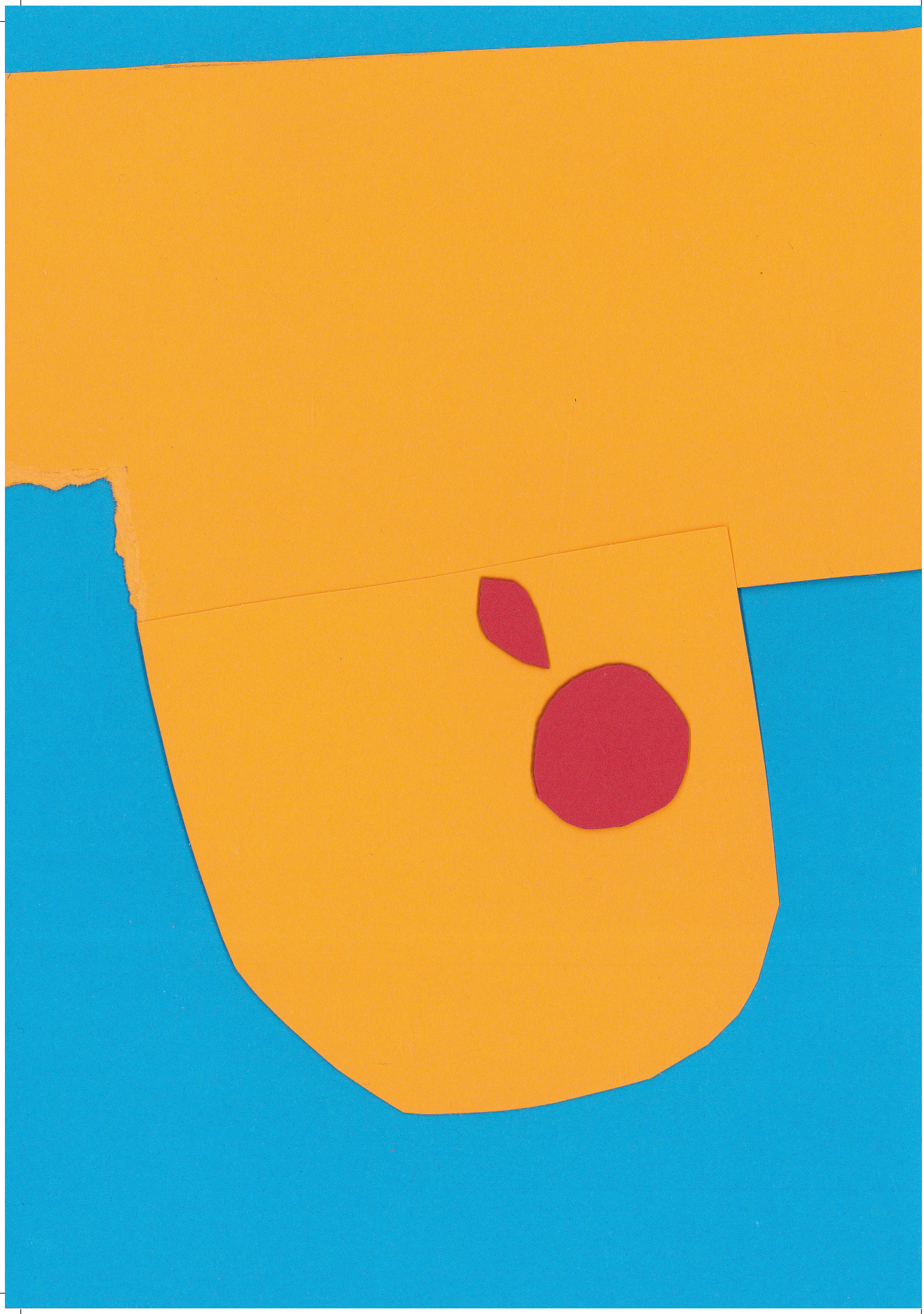

Karien_Proefschrift.indd 168 03/03/2020 11:02:04

Reference list

Aarestrup, F. M. 2015. The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. Phil. Trans. R. Soc. B 370:20140085.

Abel, G. 2015. Current status and future prospects of point-of-care testing around the globe. Expert Rev. Mol. Diagn. 15:853-855.

Abuelo, Á. and V. Alves-Nores. 2016. Point-of-care testing in cattle practice: reliabiltiy of cow-side diagnostic tests. In practice 38:293-302.

Amaral, T. and P. L. Ruegg. 2011. Association between results of Portascc, the CMT and isolation of mastitis pathogens. SMVS' Dairy Yearbook.

Andersen, S., I. R. Dohoo, R. Olde Riekerink, H. Stryhn, and a. M. R. W. Conference. 2010. Diagnosing intramammary infections: Evaluating expert opinions on the definition of intramammary infection using conjoint analysis. J. Dairy Sci. 93:2966-2975.

Appelt, S., S. S. Aly, K. Tonooka, K. Glenn, Z. Xue, T. W. Lehenbauer, and M. L. Marco. 2019. Development and comparison of loop-mediated isothermal amplification and quantitative polymerase chain reaction assays for the detection of *Mycoplasma bovis* in milk. J. Dairy Sci. 102:1985–1996.

Ashraf, A. and M. Imran. 2018. Diagnosis of bovine mastitis: from laboratory to farm. Trop Anim Health Prod 50:1193–1202.

Bager, F., F. M. Aerestrup, and H. C. Wegener. 2000. Dealing with antimicrobial resistance - the Danish experience. Can. J. Anim. Sci. 80:223-228.

Ballou, M. A. 2012. Growth and development symposium: Inflammation: Role in the etiology and pathophysiology of clinical mastitis in dairy cows. J. Anim. Sci. 90:1466–1478.

Barkema, H. W., M. A. G. von Keyserlingk, J. P. Kastelic, T. J. G. M. Lam, C. Luby, J.-P. Roy, S. J. LeBlanc, G. P. Keefe, and D. F. Kelton. 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98:7426–7445.

Barkema, H. W., Y. H. Schukken, and R. N. Zadoks. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89:1877-1895.

Barlow, J. 2011. Mastitis therapy and antimicrobial susceptibility: a multispecies review with a focus on antibiotic treatment of mastitis in cairy cattle. J Mammary Gland Biol Neoplasia 16:383–407.

Barreiro, J. R., C. R. Ferreira, G. B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M. N. Eberlin, and M. V. dos Santos. 2010. Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Dairy Sci. 93:5661–5667.

Berry, D. P. and W. J. Meaney. 2006. Interdependence and distribution of subclinical mastitis and intramammary infection among udder quarters in dairy cattle. Prev. Vet. Med. 75:81-91.

Bhutto, A. L., R. D. Murray, and Z. Woldehiwet. 2012. California mastitis test scores as indicators of subclinical intra-mammary infections at the end of lactation in dairy cows. Res. Vet. Sci. 92:13-17.

Karien_Proefschrift.indd 170 03/03/2020 11:02:04

Bradley, A. J. 2002. Bovine mastitis: an evolving disease. Vet. J. 164:116-128.

Bradley, A. J., H. Newton, and M. J. Green. 2005. Use and interpretation of bacteriology in the diagnosis of bovine intramammary infection. Mastitis in dairy production.

Brenner, H. and O. Gefeller. 1997. Variation of sensitivity, specificity, likelihood ratios and predicitive values with disease prevalence. Stat Med 16:981-991.

Busin, V., B. Wells, M. Kersaudy-Kerhoas, W. Shu, and S. T. G. Burgess. 2016. Opportunities and challenges for the application of microfluidic technologies in point-of-care veterinary diagnostics. Mol. Cell. Probes 30:331-341.

Byrt, T., J. Bishop, and J. B. Carlin. 1993. Bias, prevalence and kappa. J Clin Epidemiol 46:423-429.

Cameron, M., G. Keefe, J. Roy, I. Dohoo, K. MacDonald, and S. McKenna. 2013. Evaluation of a 3M Petrifilm on-farm culture system for the detection of intramammary infection at the end of lactation. Prev. Vet. Med. 111:1-9.

Cameron, M., S. L. McKenna, K. A. MacDonald, I. R. Dohoo, J. P. Roy, and G. P. Keefe. 2014. Evaluation of selective dry cow treatment following on-farm culture: Risk of postcalving intramammary infection and clinical mastitis in the subsequent lactation. J. Dairy Sci. 97:270-284.

Caraguel, C. G. B., H. Stryhn, N. Gagné, I. R. Dohoo, and K. L. Hammell. 2011. Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. J Vet Diagn Invest 23:2-15.

Cederlöf, S. E., N. Toft, B. Aalbaek, and I. C. Klaas. 2012. Latent class analysis of the diagnostic characteristics of PCR and conventional bacteriological culture in diagnosing intramammary infections caused by *Staphylococcus aureus* in dairy cows at dry off. Acta Vet. Scand. 54:65.

CHROMagar. 2014. CHROMagar Mastitis. Vol. 2016. 2 ed. CHROMagar.

Cornelissen, J. B. W. J., A. De Greeff, A. E. Heuvelink, M. Swarts, H. E. Smith, and F. J. van der Wal. 2016. Rapid detection of *Streptococcus uberis* in raw milk by loop-mediated isothermal amplification. J. Dairy Sci. 99:4270–4281.

Cremonesi, P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi, and M. Luzzana. 2006. Technical note: improved method for rapid DNA extraction of mastitis pathogens directly from milk. J. Dairy Sci. 89:163-169.

Cressier, B. and N. Bissonnette. 2011. Assessment of an extraction protocol to detect the major mastitis-causing pathogens in bovine milk. J. Dairy Sci. 94:2171-2184.

CRV. 2019. Beslissen van kalf tot koe. DEEL 2: MPR- en managementproducten. Vol. 2019.

de Haas, Y., H. W. Barkema, Y. H. Schukken, and R. F. Veerkamp. 2005. Associations between somatic cell count patterns and the incidence of clinical mastitis. Prev. Vet. Med. 67:55-68.

de Haas, Y., H. W. Barkema, and R. F. Veerkamp. 2002. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. J. Dairy Sci. 85:1314-1323.

Karien_Proefschrift.indd 171 03/03/2020 11:02:05

de Haas, Y., W. Ouweltjes, J. t. Napel, J. J. Windig, and G. de Jong. 2008. Alternative somatic cell count traits as mastitis indicators for genetic selection. J. Dairy Sci. 91:2501-2511.

de Haas, Y., R. F. Veerkamp, H. W. Barkema, Y. T. Gröhn, and Y. H. Schukken. 2004. Associations between pathogen-specific cases of clinical mastitis and somatic cell count patterns. J. Dairy Sci. 87:95–105.

de Koeijer, T. J., P. W. Blokland, J. F. M. Helming, H. H. Luesink, and A. van den Ham. 2014. Ex ante evaluatie wetsvoorstel Verantwoorde groei melkveehouderij : achtergronddocument. in LEI rapport. LEI Wageningen UR, Wageningen.

De Vliegher, S., L. K. Fox, S. Piepers, S. McDougall, and H. W. Barkema. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. J. Dairy Sci. 95:1025–1040.

Dingwell, R. T., K. E. Leslie, Y. H. Schukken, J. M. Sargeant, and L. L. Timms. 2003. Evaluation of the California Mastitis Test to detect an intramammary infection with a major pathogen in early lactation dairy cows. Can. Vet. J. 44:413–416.

Dohoo, I., S. Andersen, R. Dingwell, K. Hand, D. Kelton, K. Leslie, Y. Schukken, and S. Godden. 2011a. Diagnosing intramammary infections: Comparison of multiple versus single quarter milk samples for the identification of intramammary infections in lactating dairy cows. J. Dairy Sci. 94:5515-5522.

Dohoo, I. R. and K. E. Leslie. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Prev. Vet. Med. 10:225-237.

Dohoo, I. R., J. Smith, S. Andersen, D. F. Kelton, S. Godden, and Mastitis Research Workers' Conference. 2011b. Diagnosing intramammary infections: Evaluation of definitions based on a single milk sample. J. Dairy Sci. 94:250-261.

Dohoo, I. R., W. Martin, and H. Stryhn. 2009. Screening and diagnostic tests. Pages 91-134 in Veterinary Epidemiologic Research. 2nd ed. VER Inc., Charlottetown, Prince Edward Island, Canada.

Down, P. M., A. J. Bradley, J. E. Breen, and M. J. Green. 2017. Factors affecting the cost-effectiveness of on-farm culture prior to the treatment of clinical mastitis in dairy cows. Prev. Vet. Med. 145:91-99.

Duarte, C. M., P. P. Freitas, and R. Bexiga. 2015. Technological advances in bovine mastitis diagnosis: an overview. J. Vet. Diagn. Invest. 27:665-672.

Dubuc, J. 2017. Short communication: Diagnostic performance of on-farm bacteriological culture systems for identification of uterine *Escherichia coli* in postpartum dairy cows. J. Dairy Sci. 100:3079-3082.

Enøe, C., M. P. Georgiadis, and W. O. Johnsonc. 2000. Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. Prev. Vet. Med. 45:61-81.

Espetvedt, M. N., S. Rintakoski, C. Wolff, A.-K. Lind, A. Lindberg, and A.-M. K. Virtala. 2013. Nordic veterinarians' threshold for medical treatment of dairy cows, influence on disease recording and medicine use: Mild clinical mastitis as an example. Prev. Vet. Med. 112:76– 89.

Karien_Proefschrift.indd 172 03/03/2020 11:02:05

Evink, T. L. and M. I. Endres. 2017. Management, operational, animal health, and economic characteristics of large dairy herds in 4 states in the Upper Midwest of the United States. J. Dairy Sci. 100:9466-9475.

Ferreira, J. C., M. S. Gomes, E. C. R. Bonsaglia, I. F. Canisso, E. F. Garrett, J. L. Stewart, Z. Zhou, and F. S. Lima. 2018. Comparative analysis of four commercial on-farm culture methods to identify bacteria associated with clinical mastitis in dairy cattle. PLoS ONE 13(3):e0194211.

Fleiss, J. L. 1971. Measuring nominal scale agreement among many raters. Physiological Bulletin 76:378-382.

Formularium. 2016. Formularium Melkvee. Werkgroep veterinair antibioticumbeleid, ed. Koninklijke Nederlandse Maatschappij voor Diergeneeskunde.

Fosgate, G. T., I.M. Petzer, and J. Karzis. 2013. Sensitivity and specificity of a hand-held milk electrical conductivity meter compared to the California Mastitis Test for mastitis in dairy cattle. Vet. J. 196:98-102.

Friggens, N. C., M. G. G. Chagunda, M. Bjerring, C. Ridder, S. Højsgaard, and T. Larsen. 2007. Estimating degree of mastitis from time-series measurements in milk: A test of a model based on lactate dehydrogenase measurements. J. Dairy Sci. 90:5415–5427.

Fuenzalida, M. J. and P. L. Ruegg. 2019. Negative controlled, randomized clinical trial to evaluate intramammary treatment of nonsevere, gram-negative clinical mastitis. J. Dairy Sci. 102:5438-5457.

Ganda, E. K., R. S. Bisinotto, D. H. Decter, and R. C. Bicalho. 2016. Evaluation of an on-farm culture system (Accumast) for fast identification of milk pathogens associated with clinical mastitis in dairy cows. PloS ONE 11:1-16.

van Geijlswijk, I. M., D. J. J. Heederik, J. W. Mouton, J. A. Wagenaar, J. H. Jacobs, and I. P. Sanders. 2019. Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2018 - Trends and benchmarking of livestock farms and veterinarians. Authoriteit Diergeneesmiddelen (SDa).

Green, M. J., L. E. Green, Y. H. Schukken, A. J. Bradley, E. J. Peeler, H. W. Barkema, Y. de Haas, V. J. Collis, and G. F. Medley. 2004. Somatic cell count distributions during lactation predict clinical mastitis. J. Dairy Sci. 87:1256-1264.

Griffioen, K., G. E. Hop, M. M. C. Holstege, A. G. J. Velthuis, T. J. G. M. Lam, and 1Health4Food Mastitis Diagnostic Consortium. 2016. Dutch dairy farmers' need for microbiological mastitis diagnostics. J. Dairy Sci. 99:5551–5561.

Griffioen, K., A. G. J. Velthuis, L. A. Lagerwerf, A. E. Heuvelink, and T. J. G. M. Lam. 2018. Agreement between four commercial diagnostic tests and routine bacteriological culture of milk to determine the udder infection status of dairy cows. Prev. Vet. Med. 157:162-173.

Guion, C. E., T. J. Ochoa, C. M. Walker, F. Barletta, and T. G. Cleary. 2008. Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. J. Clin. Microbiol. 46:1752-1757.

Karien_Proefschrift.indd 173 03/03/2020 11:02:05

Gurjar, A., G. Gioia, Y. H. Schukken, F. Welcome, R. Zadoks, and P. Moroni. 2012. Molecular diagnostics applied to mastitis problems on dairy farms. Vet. Clin. North Am. Food Anim. Pract. 28:565-576.

Guterbock, W. M., A. L. van Eenennaam, R. J. Anderson, I. A. Gardner, J. S. Cullor, and C. A. Holmberg. 1993. Efficacy of intramammary antibiotic therapy for treatment of clinical mastitis caused by environmental pathogens. J. Dairy Sci. 76:3437-3444.

Halasa, T., M. Nielen, A. C. Whist, and O. Østerås. 2009. Meta-analysis of dry cow management for dairy cattle. Part 2. Cure of existing intramammary infections. J. Dairy Sci. 92:3150-3157.

Hawkins, D. 2019. Use of different somatic cell count cut-points to define intramammary infection at drying off in dairy cows from a herd with a high somatic cell count. N Z Vet J 67(4):203-209.

Hiitiö, H., S. Pyörälä, S. Taponen, P. Rajala-Schultz, and H. Simojoki. 2018. Elimination of experimentally induced bovine intramammary infection assessed by multiplex real-time PCR and bacterial culture. J. Dairy Sci. 101:1-10.

Hiitiö, H., R. Riva, T. Autio, T. Pohjanvirta, J. Holopainen, S. Pyörälä, and S. Pelkonen. 2015. Performance of a real-time PCR assay in routine bovine mastitis diangostics compared with in-depth conventional culture. J. Dairy Res. 82:200-208.

Hiitiö, H., H. Simojoki, P. Kalmus, S. Pyörälä, and S. Taponen. 2016. The effect of sampling technique on PCR-based bacteriological results of bovine milk samples. J. Dairy Sci. 99:6532-6541.

Hill, A. E., A. L. Green, B. A. Wagner, and D. A. Dargatz. 2009. Relationship between herd size and annual prevalence of and primary antimicrobials treatments for common diseases on dairy operations in the United States. Prev. Vet. Med. 88:264-277.

Hillerton, J. and E. Berry. 2005. Treating mastitis in the cow–a tradition or an archaism. J. Appl. Microbiol. 98:1250-1255.

Hiss, S., U. Mueller, A. Neu-Zahren, and H. Sauerwein. 2007. Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters. Vet. Med. 52:245-252.

Hoe, F. G. H. and P. L. Ruegg. 2006. Opinions and practices of wisconsin dairy producers about biosecurity and animal well-being. J. Dairy Sci. 89:2297–2308.

Hogeveen, H., C. Kamphuis, W. Steeneveld, and H. Mollenhorst. 2010. Sensors and Clinical Mastitis—The Quest for the Perfect Alert. Sensors 10:7991-8009.

Hou, P., H. Wang, G. Zhao, C. He, and H. He. 2017. Rapid detection of infectious bovine Rhinotracheitis virus using recombinase polymerase amplification assays. BMC Vet. Res. 13:386.

Huckle, D. 2015. Point-of-care diagnostics: an advancing sector with nontechnical issues. Expert Rev. Mol. Diagn. 8:679-688.

Hui, S. L. and S. D. Walter. 1980. Estimating the error rates of diagnostic tests. Biometrics 36:167-171.

Jansen, J., B. H. P. van den Borne, R. J. Renes, G. van Schaik, T. Lam, and C. Leeuwis. 2009. Explaining mastitis incidence in Dutch dairy farming: The influence of farmers' attitudes and behaviour. Prev. Vet. Med. 92:210-223.

Karien_Proefschrift.indd 174 03/03/2020 11:02:05

Jashari, R., S. Piepers, and S. De Vliegher. 2016. Evaluation of the composite milk somatic cell count as a predictor of intramammary infection in dairy cattle. J. Dairy Sci. 99:9271-9286.

Jones, G., O. Bork, S. A. Ferguson, and A. Bates. 2019. Comparison of an on-farm point-of-care diagnostic with conventional culture in analysing bovine mastitis samples. J. Dairy Res. 86:222-225.

Kamphuis, C., H. Mollenhorst, and H. Hogeveen. 2011. Sensor measurements revealed: Predicting the Gram-status of clinical mastitis causal pathogens. Comput Electron Agric 77:86-94.

Kaneko, H., T. Kawana, E. Fukushima, and T. Suzutani. 2007. Tolerance of loop-mediated isothermal amplifiaction to a culture medium and biological substances. J. Biochem. Biophys. Methods 70:499-501.

Kayitsinga, J., R. L. Schewe, G. A. Contreras, and R. J. Erskine. 2016. Antimicrobial treatment of clinical mastitis in the eastern United States: The influence of dairy farmers' mastitis management and treatment behavior and attitudes. J. Dairy Sci. 100:1388-1407.

Keefe, G., K. MacDonald, and M. Cameron. 2013. On-farm culture: Role in mastitis and impact on antimicrobial use. eXtension.

Koskinen, M. T., J. Holopainen, S. Pyorala, P. Bredbacka, A. Pitkala, H. W. Barkema, R. Bexiga, J. Roberson, L. Solverod, R. Piccinini, D. Kelton, H. Lehmusto, S. Niskala, and L. Salmikivi. 2009. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. J. Dairy Sci. 92:952-959.

Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W. A. van Haeringen, T. J. G. M. Lam, and S. Pyörälä. 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J. Dairy Sci. 93:5707-5715.

Krogh, M. A., N. Toft, and C. Enevoldsen. 2011. Latent class evaluation of a milk test, a urine test, and the fat-to-protein percentage ratio in milk to diagnose ketosis in dairy cows. J. Dairy Sci. 94:2360–2367.

Krömker, V. and S. Leimbach. 2017. Mastitis treatment—Reduction in antibiotic usage in dairy cows. Reprod. Dom. Anim. 52 (Suppl. 3):21-29.

Kuipers, A., W. J. Koops, and H. Wemmenhove. 2015. Antibiotic use in dairy herds in the Netherlands from 2005 to 2012 J. Dairy Sci. 99:1632-1648.

Lago, A., S. M. Godden, R. Bey, P. L. Ruegg, and K. Leslie. 2011a. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. J. Dairy Sci. 94:4441–4456.

Lago, A., S. M. Godden, R. Bey, P. L. Ruegg, and K. Leslie. 2011b. The selective treatment of clinical mastitis based on on-farm culture results: II. Effects on lactation performance, including clinical mastitis recurrence, somatic cell count, milk production, and cow survival. J. Dairy Sci. 94:4457-4467.

Lam, T. J. G. M., J. Jansen, and R. J. Wessels. 2017. The RESET mindset model applied on decreasing antibiotic usage in dairy cattle in the Netherlands. Ir Vet J 70.

Karien_Proefschrift.indd 175 03/03/2020 11:02:06

Lam, T. J. G. M., R. G. M. Olde Riekerink, O. C. Sampimon, and H. Smith. 2009. Mastitis diagnostics and performance monitoring: a practical approach. Ir Vet J 62:34-39.

Lam, T. J. G. M., B. H. P. van den Borne, J. Jansen, K. Huijps, J. C. L. van Veersen, G. van Schaik, and H. Hogeveen. 2013. Improving bovine udder health: a national mastitis control program in the Netherlands. J. Dairy Sci. 96:1301-1311.

Landis, J. R. and G. G. Koch. 1977. The measurement of observer agreement for categorical data. Biometrics:159-174.

Lee, S., V. S. L. Khoo, C. A. D. Medriano, T. Lee, S.-Y. Park, and S. Bae. 2019. Rapid and in-situ detection of fecal indicator bacteria in water using simple DNA extraction and portable loop-mediated isothermal amplification (LAMP) PCR methods. Water Res. 160:371-379.

Leimbach, S. and V. Krömker. 2018. Laboratory evaluation of a novel rapid tube test system for differentiation of mastitis-causing pathogen groups. J. Dairy Sci. 101:1-9.

Leslie, K., M. Walker, E. Vernooy, A. Bashiri, and R. Dingwell. 2005. Evaluation of the Petrifilm™ culture system for the identification of mastitis bacteria as compared to standard bacteriological methods. Mastitis in dairy production.

Levy, S. B. and B. Marshall. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10:S122-S129.

Li, J. and J. P. Fine. 2011. Assessing the dependence of sensitivity and specificity on prevalence in meta-analysis. Biostatistics:kxr008.

Likert, R. 1932. A technique for the measurement of attitudes. Arch. Psychol. 140:1-55.

Looney, S. W. and J. L. Hagan. 2008. Statistical methods for assessing biomarkers and analyzing biomarker data. Vol. 27. Handbook of statistics: Epidemiology and medical statistics.

Lunn, D., D. Spiegelhalter, A. Thomas, and N. Best. 2009. The BUGS project: Evolution, critique and future directions. Stat Med 28:3049–3067.

MacDonald, K. A., G. P. Keefe, I. Dohoo, K. Leslie, and J. P. Roy. 2011. Promoting judicious antibiotic use: On-farm culture-based treatement strategies. In Udder Health and Communcation. H. Hogeveen and T. J. G. M. Lam, ed. Wageningen Academic Publishers,Wageningen.

Madoz, L. V., I. Prunner, M. Jaureguiberry, C. C. Gelfert, R. L. de la Sota, M. J. Giuliodori, and M. Drillich. 2017. Application of a bacteriological on-farm test to reduce antimicrobial usage in dairy cows with purulent vaginal discharge. J. Dairy Sci. 100:3875-3882.

Mahmmod, Y. S., N. Toft, J. Katholm, C. Gronbaek, and I. C. Klaas. 2013a. Estimation of test characteristics of real-time PCR and bacterial culture for diagnosis of subclinical intramammary infections with *Streptococcus agalactiae* in Danish dairy cattle in 2012 using latent class analysis. Prev. Vet. Med. 109:264-270.

Karien_Proefschrift.indd 176 03/03/2020 11:02:06

Mahmmod, Y. S., N. Toft, J. Katholm, C. Grønbæk, and I. C. Klaas. 2013b. Bayesian estimation of test characteristics of real-time PCR, bacteriological culture and California Mastitis test for diagnosis of intramammary infections with *Staphylococcus aureus* in dairy cattle at routine milk recordings. Prev. Vet. Med. 112:309-317.

Mansion-de Vries, E. M., N. Knorr, J. H. Paduch, C. Zinke, M. Hoedemaker, and V. Krömker. 2014. A field study evaluation of Petrifilm™ plates as a 24-h rapid diagnostic test for clinical mastitis on a dairy farm. Prev. Vet. Med. 113:620-624.

McCarron, J., G. Keefe, S. McKenna, I. Dohoo, and D. Poole. 2009. Laboratory evaluation of 3M Petrifilms and University of Minnesota Bi-plates as potential on-farm tests for clinical mastitis. J. Dairy Sci. 92:2297-2305.

Menten, J., M. Boelaert, and E. Lesaffre. 2008. Bayesian latent class models with conditionally dependent diagnostic tests: A case study. Stat Med 27:4469–4488.

Milner, P., K. Page, and J. Hillerton. 1997. The effects of early antibiotic treatment following diagnosis of mastitis detected by a change in the electrical conductivity of milk. J. Dairy Sci. 80:859-863.

Minnesota, U. o. 2013. Minnesota Easy Culture System User's Guide.

Monis, P. T., S. Giglio, and C. P. Saint. 2005. Comparison of SYT09 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem. 340:24-34.

Mori, Y. and T. Notomi. 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15:62-69.

Neeser, N. L., W. D. Hueston, S. M. Godden, and R. F. Bey. 2006. Evaluation of the use of an on-farm system for bacteriological culture of milk from cows with low-grade mastitis. J. Am. Vet. Med. Assoc. 228:254-260.

Nielen, M., H. Deluyker, Y. H. Schukken, and A. Brand. 1992. Electrical conductivity of milk: Measurement, modifiers, and meta analysis of mastitis detection performance. J. Dairy Sci. 75:606-614.

NMC. 1999. Laboratory handbook on bovine mastitis. NMC Inc., Madison.

NMC. 2016. Current concepts of bovine mastitis. Fifth ed.

Nyman, A.-K., U. Emanuelson, and K. P. Waller. 2016. Diagnostic test performance of somatic cell count, lactate dehydrogenase, and N-acetyl-β-D-glucosaminidase for detecting dairy cows with intramammary infection. J. Dairy Sci. 99:1440-1448.

Oikonomou, G., V. S. Machado, C. Santisteban, Y. H. Schukken, and R. C. Bicalho. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16S rDNA. PLoS ONE 7:e47671.

Olde Riekerink, R. G. M., H. W. Barkema, D. F. Kelton, and D. T. Scholl. 2008. Incidence rate of clinical mastitis on Canadian dairy rarms. J. Dairy Sci. 91:1366–1377.

Owens, W. E., C. H. Ray, J. L. Watts, and R. J. Yancey. 1997. Comparison of success of antibiotic therapy during lactation and results of antibmicrobial susceptibility tests for bovine mastitis. J. Dairy Sci. 80:313-317.

Karien_Proefschrift.indd 177 03/03/2020 11:02:06

Pennings, J. M. E., S. H. Irwin, and D. L. Good. 2002. Surveying farmers: a case study. Review of Agricultural Economics 24:266-277.

Petzer, I.-M., J. Karzis, E. F. Donkin, E. C. Webb, and E. M. C. Etter. 2017. Validity of somatic cell count as indicator of pathogen-specific intramammary infections. J S Afr Vet Assoc 88:a1465.

Piepers, S., L. D. Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. De Vliegher. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J Dairy Res. 74:478-483.

Pinzón-Sánchez, C., V. Cabrera, and P. Ruegg. 2011. Decision tree analysis of treatment strategies for mild and moderate cases of clinical mastitis occurring in early lactation. J. Dairy Sci. 94:1873-1892.

Pinzón-Sánchez, C. and P. L. Ruegg. 2011. Risk factors associated with short-term post-treatment outcomes of clinical mastitis. J. Dairy Sci. 94:3397-3410.

Pol, M. and P. L. Ruegg. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J. Dairy Sci. 90:249-261.

Pyörälä, S. 2003. Indicators of inflammation in the diagnosis of mastitis. Vet. Res. 34:565–578.

Pyörälä, S. 2009. Treatment of mastitis during lactation. Ir Vet J 62:40-44.

Reed, G. H., J. O. Kent, and C. T. Wittwer. 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics 8:597-608.

Reksen, O., L. Sølverød, and O. Østeras. 2008. Relationships between milk culture results and composite milk somatic cell counts in Norwegian dairy cattle. J. Dairy Sci. 91:3102–3113.

Roberson, J. R. 2003. Establishing treatment protocols for clinical mastitis. Vet. Clin. North Am. Food Anim. Pract. 19:223-234.

Roberson, J. R. 2012. Treatment of clinical mastitis. Vet. Clin. North Am. Food Anim. Pract. 28:271–288.

Royster, E., S. Godden, D. Goulart, A. Dahlke, P. Rapnicki, and J. Timmerman. 2014. Evaluation of the Minnesota Easy Culture System II Bi-Plate and Tri-Plate for identification of common mastitis pathogens in milk. J. Dairy Sci. 97:3648-3659.

Royster, E. and S. Wagner. 2015. Treatment of mastitis in cattle. Vet Clin Food Anim 31:17-46.

Ruegg, P. 2018. Making antibiotic treatment decisions for clinical mastitis. Vet. Clin. North Am. Food Anim. Pract. 34:413–425.

Ruegg, P., S. Godden, A. Lago, and R. Bey. 2009. On-farm culturing for better milk quality. Pages 149-159 in Proc. Proceedings of 2009 Western Dairy Management Conference. Kansas State University Manhattan (KS).

Ruegg, P. L. and D. J. Reinemann. 2002. Milk quality and mastitis tests.

Rutten, C. J. 2017. The utility of sensor technology to support reproductive management on dairy farms. Wageningen University.

Karien_Proefschrift.indd 178 03/03/2020 11:02:06

Sampimon, O. C., H. W. Barkema, I. Berends, J. Sol, and T. Lam. 2009. Prevalence of intramammary infection in Dutch dairy herds. J. Dairy Res. 76:129-136.

Sandford, C. J., G. P. Keefe, J. Sanchez, R. T. Dingwell, H. W. Barkema, K. E. Leslie, and I. R. Dohoo. 2006. Test characteristics from latent-class moders of the California Mastitis Test. Prev. Vet. Med. 77:96-108.

Sange, M. D., A. Becker, A. A. Hassan, M. Bülte, M. Ganter, U. Siebert, and A. Abdulmawjood. 2019. Development and validation of a loop-mediated isothermal amplification assay—a rapid and sensitive detection tool for *Mycobacterium avium* subsp. paratuberculosis in small ruminants. J. Appl. Microbiol. 127:47-58.

Santman-Berends, I. M. G. A., T. J. G. M. Lam, J. Keurentjes, and G. van Schaik. 2015. An estimation of the clinical mastitis incidence per 100 cows per year based on routinely collected herd data. J. Dairy Sci. 98:6965-6977.

Santman-Berends, I. M. G. A., J. M. Swinkels, T. J. G. M. Lam, J. Keurentjes, and G. van Schaik. 2016. Evaluation of udder health parameters and risk factors for clinical mastitis in Dutch dairy herds in the context of a restricted antimicrobial usage policy. J. Dairy Sci. 99:2930-2939.

Sargeant, J. M., K. E. Leslie, J. E. Shirley, B. J. Pulkrabek, and G. H. Lim. 2001. Sensitivity and specificity of somatic cell count and California Mastitis Test for identifying intramammary infection in early lactation. J. Dairy Sci. 84:2018–2024.

Sathish, G., E. Hemakumar, and K. C. Divya. 2016. Rapid detection of MRSA by loop-mediated isothermal amplification in bovine milk samples. Br Microbiol Res J 17:1-5.

Scherpenzeel, C. G. M., S. H. W. Tijs, I. E. M. den Uijl, I. M. G. A. Santman-Berends, A. G. J. Velthuis, and T. J. G. M. Lam. 2016. Farmers' attitude toward the introduction of selective dry cow therapy. J. Dairy Sci. 99:8259-8266.

Scherpenzeel, C. G. M., E. M. den Uijl, G. van Schaik, R. G. M. O. Riekerink, J. M. Keurentjes, and T. J. G. M. Lam. 2014. Evaluation of the use of dry cow antibiotics in low somatic cell count cows. J. Dairy Sci. 97:3606-3614.

Schmenger, A., S. Leimbach, and V. Krömker. 2018. Introducing an evidence-based mastitis therapy concept to a conventional dairy farm. Pages 58-62 in Proc. One health and food safety, Bonn, Germany.

Schukken, Y. H., D.J. Wilson, F. Welcome, L. Garrison-Tikofsky, and R. N. Gonzale. 2003. Monitoring udder health and milk quality using somatic cell counts. Vet. Res. 34:579-596.

Schukken, Y. H., F. J. Grommers, J. A. Smit, D. Vandegeer, and A. Brand. 1989. Effect of freezing on bacteriologic culturing of mastitis milk samples. J. Dairy Sci. 72:1900-1906.

Sears, P. M. and K. K. McCarthy. 2003. Diagnosis of mastitis for therapy decisions. Vet. Clin. North Am. Food Anim. Pract. 19:93-108.

Sears, P. M., B. S. Smith, P. B. English, P. S. Herer, and R. N. Gonzalez. 1990. Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. J. Dairy Sci. 73:2785-2789.

Sheet, O. H., N. T. Grabowski, G. Klein, and A. Abdulmawjood. 2016. Development and validation of a loop mediated isothermal amplification (LAMP) assay for the detection of *Staphylococcus aureus* in bovine mastitis milk samples. Mol. Cell. Probes 30:320-325.

Karien_Proefschrift.indd 179 03/03/2020 11:02:06

Shome, B. R., S. D. Mitra, M. Bhuvana, N. Krithiga, D. Velu, R. Shome, S. Isloor, S. B. Barbuddhe, and H. Rahman. 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. J. Appl. Microbiol. 1349-1356.

Sim, J. and C. C. Wright. 2005. The kappa statistic in reliability studies: Use, interpretation, and sample size requirements. Phys Ther 85:257-268.

Sol, J., O. C. Sampimon, E. Hartman, and H. W. Barkema. 2002. Effect of preculture freezing and incubation on bacteriological isolation from subclinical mastitis samples. Vet. Microbiol. 85:241-249.

Song, L., J. Li, S. Hou, X. Li, and S. Chen. 2012. Establishment of loop-mediated isothermal amplification (LAMP) for rapid detection of *Brucella* spp. and application to milk an blood samples. J. Microbiol. Methods 90:292-297.

Sowmya, N., M. S. Thakur, and H. K. Manonmani. 2012. Rapid and simple DNA extraction method for the detection of enterotoxigenic *Staphylococcus aureus* directly from food samples: comparison of PCR and LAMP methods. J. Appl. Microbiol. 113:106-113.

Speksnijder, D. C., D. J. Mevius, C. J. M. Bruschke, and J. A. Wagenaar. 2015. Reduction of veterinary antimicrobial use in the Netherlands. The Dutch success model. Zoonoses Public Health 62:79-87.

St-Louis, P. 2000. Status of point-of-care testing: promise, realities, and possibilities. Clin. Biochem. 33:427-440.

Suojala, L., H. Simojoki, K. Mustonen, L. Kaartinen, and S. Pyörälä. 2010. Efficacy of enrofloxacin in the treatment of naturally occurring acute clinical *Escherichia coli* mastitis. J. Dairy Sci. 93:1960–1969.

Suriyasathaporn, W., Y. H. Schukken, M. Nielen, and A. Brands. 2000. Low somatic cell count: a risk factor for sebsequent clinical mastitis in a dairy herd. J. Dairy Sci. 83:1248-1255.

Swinkels, J. M., P. Cox, Y. H. Schukken, and T. J. G. M. Lam. 2013. Efficacy of extended cefquinome treatment of clinical *Staphylococcus aureus* mastitis. J. Dairy Sci. 96:4983–4992.

Swinkels, J. M., A. Hilkens, V. Zoche-Golob, V. Krömker, M. Buddiger, J. Jansen, and T. J. G. M. Lam. 2015. Social influences on the duration of antibiotic treatment of clinical mastitis in dairy cows. J. Dairy Sci. 98:2369-2380.

Tangkanchanapasa, P., M. Höfte, and K. D. Jonghe. 2018. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) designed for fast and sensitive on-site detection of Pepper chat fruit viroid (PCFVd). J. Virol. Methods 259:81-91.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä. 2010. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J. Dairy Sci. 92:2610-2617.

Tatone, E. H., J. L. Gordon, J. Hubbs, S. J. LeBlanc, T. J. DeVries, and T. F. Duffield. 2016. A systematic review and meta-analysis of the diagnostic accuracy of point-of-care tests for the detection of hyperketonemia in dairy cows. Prev. Vet. Med. 130:18-32.

Tian, X., J. Feng, and Y. Wang. 2018. Direct loop-mediated isothermal amplification assay for on-site detection of *Staphylococcus aureus*. FEMS Microbiol. Lett. 365:1-6.

Karien_Proefschrift.indd 180 03/03/2020 11:02:07

Tie, Z., W. Chunguang, W. Xiaoyuan, Z. Xingua, and Z. Xiuhui. 2012. Loop-mediated isothermal amplification for detection of *Staphylococcus aureus* in dairy cow suffering from mastitis. J. Biomed. Biotechnol.:5.

Toft, N., E. Jørgensen, and S. Højsgaard. 2005. Diagnosing diagnostic tests: evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. Prev. Vet. Med. 68:19-33.

Vaarst, M., B. Paarup-Laursen, H. Houe, C. Fossing, and H. Andersen. 2002. Farmers' choice of medical treatment of mastitis in Danish dairy herds based on qualitative research interviews. J. Dairy Sci. 85:992-1001.

van den Borne, B. H., G. van Schaik, T. J. Lam, and M. Nielen. 2010. Therapeutic effects of antimicrobial treatment during lactation of recently acquired bovine subclinical mastitis: Two linked randomized field trials. J. Dairy Sci. 93:218-233.

van den Borne, B. H. P., J. C. M. Vernooij, A. M. Lupindu, G. van Schaik, K. Franken, T. J. G. M. Lam, and M. Nielen. 2011. Relationship between somatic cell count status and subsequent clinical mastitis in Dutch dairy cows. Prev. Vet. Med. 102:265- 273.

Vanholder, T. and M. Melchior. 2012. Mastitis bij Melkkoeien. Vol. 59. 1 ed. Diergeneeskundig Memorandum. Stichting Diergeneeskundig Memorandum, Nijmegen.

Vanhoudt, A., K. van Hees - Huijps, A. T. M. van Knegsel, O. C. Sampimon, J. C. M. Vernooij, M. Nielen, and T. van Werven. 2018. Effects of reduced intramammary antimicrobial use during the dry period on udder health in Dutch dairy herds. J. Dairy Sci. 101:1-13.

Vasquez, A. K., C. Foditsch, S.-A. C. Dulièpre, J. D. Siler, D. R. Just, L. D. Warnick, D. V. Nydam, and J. Sok. 2019. Understanding the effect of producers' attitudes, perceived norms, and perceived behavioral control on intentions to use antimicrobials prudently on New York dairy farms. PLoS ONE 14:e0222442.

Vasquez, A. K., D. V. Nydam, C. Foditsch, M. Wieland, R. Lynch, S. Eicker, and P. D. Virkle. 2018. Use of a culture-independent on-farm algorithm to guide the use of selective dry-cow antibiotic therapy J. Dairy Sci. 101:5345-5361.

Viguier, C., S. Arora, N. Gilmartin, K. Welbeck, and R. O'Kennedy. 2009. Mastitis detection: current trends and future perspectives. Trends Biotechnol. 27:486-493.

Viora, L., E. Graham, D. Mellor, K. Reynolds, P. Simoes, and T. Geraghty. 2014. Evaluation of a culture-based pathogen identification kit for bacterial causes of bovine mastitis. Vet. Rec. 175:89-89.

Wallace, J. 2011. On-farm mastitis diagnosis.

Wang, D. and Y. Li. 2015. Development of primer sets for loop-mediated isothermal amplification that enables rapid and specific detection of *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Streptococcus agalactiae*. Int J Environ Res Public Health 20:5735-5742.

Wang, Z., N. Dendukuri, and L. Joseph. 2017. Understanding the effects of conditional dependence in research studies involving imperfect diagnostic tests. Stat Med 36:466-480.

Watson, P. F. and A. Petrie. 2010. Method agreement analysis: A review of correct methodology. Theriogenology 73:1167-1179.

Karien_Proefschrift.indd 181 03/03/2020 11:02:07

Watts, J. L. 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16:41-66.

Wilisiani, F., A. Tomiyama, H. Katoh, S. Hartono, Y. Neriya, H. Nishigawa, and T. Natsuaki. 2019. Development of a LAMP assay with a portable device for real-time detection of begomoviruses under field conditions. J. Virol. Methods 256:71-76.

Wilkins, J. R., S. M. Mills, and E. H. Boykin. 1972. Automatic surface inoculation of agar trays. J. Appl. Microbiol. 24:778-785.

Yang, W., X. Song, J. Wang, Z. Li, M. Ji, and Y. Li. 2014. Detection methods for milk pathogenic bacteria by loop-mediated isothermal amplification. Biosci Trends 8:316-321.

Yang, Z., G. Xu, J. Reboud, S. A. Ali, G. Kaur, J. McGiven, N. Boby, P. K. Gupta, P. Chaudhuri, and J. M. Cooper. 2018. Rapid veterinary diagnosis of bovine reproductive infectious diseases from semen using paper-origami DNA microfluidics. ACS Sens 3:403−409.

Zadoks, R. N., H. G. Allore, H. W. Barkema, O. C. Sampimon, G. J. Wellenberg, Y. T. Gröhn, and Y. H. Schukken. 2001. Cow- and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. J. Dairy Sci. 84:2649-2663.

Zadoks, R. N., H. M. Griffiths, M. A. Munoz, C. Ahlstrom, G. J. Bennett, E. Thomas, and Y. H. Schukken. 2011. Sources of Klebsiella and Raoultella species on dairy farms: Be careful where you walk. J. Dairy Sci. 94:1045-1051.

Zakian, A., M. Tehrani-Sharif, M. R. Mokhber-Dezfouli, M. Nouria, and P. D. Constable. 2017. Evaluation of a point-of-care electrochemical meter to detect subclinical ketosis and hypoglycaemia in lactating dairy cows. Aust. Vet. J. 95:123-128.

Zamani, A., R. Y. Mashouf, A. E. Namvar, and M. Y. Alikhani. 2013. Detection of magA gene in *Klebsiella* spp. isolated from clinical samples. Iran J Basic Med Sci 13:173-176.

Karien_Proefschrift.indd 182 03/03/2020 11:02:07

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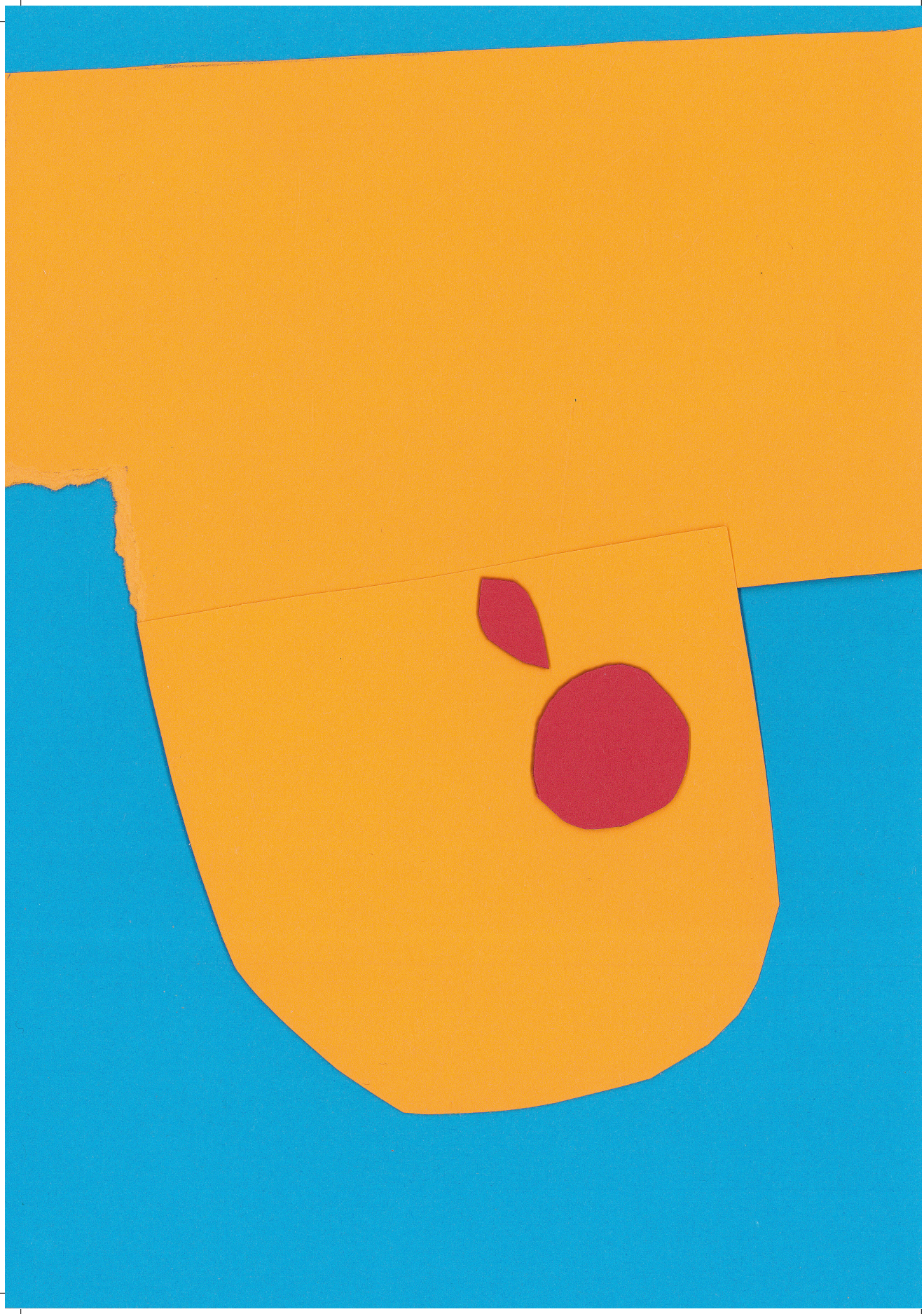

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Summary

Testing at the point-of-care (POC) means that a diagnostic test is performed near the patient as opposed to testing in a laboratory. Both in human and veterinary medicine POC tests are increasingly used. One of the reasons is the reduced time between sample collection and test result, which enables quicker diagnosis and can help to optimize treatments.

Mastitis (inflammation of the udder) is a disease in cows that contributes most to the amount of antimicrobials used at dairy farms. Knowledge on the cause of mastitis might help to target treatments and to reduce the number of antimicrobial treatments. Furthermore, targeted treatments may reduce the potential effect of antimicrobial use on antimicrobial resistance development of causative bacteria. Application in other countries showed that the use of mastitis diagnostic tests leads to an increased percentage of the antimicrobial treatments being targeted as compared to if such tests were not used. Even though tests are available to assist in using antimicrobials prudently, they are not frequently used by Dutch farmers for mastitis treatment decisions. Since farmers often question mastitis treatment decisions, there may be a need among Dutch dairy farmers for additional information on the cause of mastitis, in order to facilitate treatment decisions and to enhance prudent antimicrobial use for mastitis.

The aim of this thesis was to determine the added value of diagnostic information on the mastitis treatment strategy of Dutch dairy farmers. The ultimate goal is to contribute to improved mastitis treatments on dairy farms to enhance prudent antimicrobial use for mastitis.

The need for reliable diagnostic mastitis tests among Dutch dairy farmers was captured during a telephonic questionnaire, which is described in *chapter 2*. For clinical mastitis, the main interest was for a test resulting in an advice on the antimicrobial to use, whereas for subclinical mastitis interviewees were mainly interested in the causative bacterium. Twice as many farmers would use tests for treatment decisions as compared to the number of farmers who currently use diagnostics for treatment decisions on clinical mastitis, subclinical mastitis and drying off, if such a test has a suitable time-to-result and is available for on-farm use.

Chapter 3 reports the results of a laboratory evaluation of four commercial culture-based tests that can be used on dairy farms. These tests were compared for their ability to detect mastitis pathogens in milk samples routinely submitted to a laboratory. All four

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tests were more or less equally able to detect presumptive mastitis-causing pathogens from three diagnostic categories (Gram-positive, Gram-negative, and no growth). Thus, all tests could be used for generating treatment advice, and none of the tests can be designated as the optimal test.

The time-to-result of culture-based tests is generally 24h, which does not match with the wishes of dairy farmers. *Chapter 4* describes the development and evaluation of four loop-mediated isothermal amplification (LAMP) assays which have a short time-to-result. These assays, detecting *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus* spp., were applied to isolates and field samples. Also, a nucleic acid lateral flow immunoassay (NALFIA) was evaluated as simple read-out, suitable for on-site use. An almost perfect agreement (≥ 0.80) was found as compared to the reference method, except for the *Streptococcus* spp. assay, which had lower agreement. The assays might be promising for fast on-site tests to detect mastitis-causing pathogens, provided a fast and simple method for DNA isolation becomes available.

In *chapter 5* the results of a telephone questionnaire and a field study are described about the added value of diagnostic information at dry-off. The questionnaire revealed the need of Dutch dairy farmers for additional tests at dry-off and their interest in quarter level treatment decisions as a step to further reduce antimicrobial usage. In the field study, diagnostic information at the quarter level was provided to the farmers, in addition to the current available information at dry-off. They used this quarter level information, but made cow level decisions. Adding diagnostic information resulted in farmers treating more quarters with antimicrobials than they were used to do in their current approach, but treatments were more targeted. Selective use of diagnostic information for cows with high somatic cell count at dry-off might be the approach to optimize dry-cow treatment decisions, but needs further research.

Chapter 6 gives the results of a field study in which farmers used two culture-based tests to decide on treatment of clinical and subclinical mastitis. Cure and antimicrobial use were compared to the current treatment farmers apply in case of clinical or subclinical mastitis. Adding diagnostic information resulted in more treatments applied in accordance with the cause of mastitis for both clinical and subclinical mastitis. Antimicrobial usage was lower or equal for clinical mastitis, but higher in case of subclinical mastitis, as compared to the current approach of farmers. Cure was similar or

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higher if farmers applied their current approach, suggesting that they include important predictive information when deciding on treatment, which is not captured in a result of the on-farm tests used.

Because bacteriological culture is not a perfect reference test, *chapter 7* reports on the evaluation of two culture-based tests using latent class analysis. Both tests were subjected to a set of mastitis field samples, and cultured and read in the laboratory as well as by farmers on-farm. Three latent statuses were evaluated, Gram-positive bacteria, Gram-negative bacteria, and no growth. One test was less sensitive to detect Gram-positive bacteria if read on-farm. In general, tests used in the laboratory seemed to perform better than those used on-farm.

In *chapter 8* the main results and conclusions of this thesis are discussed. Farmers have a positive mindset towards prudent antimicrobial use for mastitis. They consider diagnostic information as of added value to the current clinical mastitis treatment strategy. On-farm tests can help to categorize mastitis cases into one of three diagnostic categories, Gram-positive, Gram-negative, or no growth, which enables farmers to optimize mastitis treatment decisions in terms of applying antimicrobials targeted at the detected presumptive mastitis-causing pathogen. However, if the current treatment strategy of farmers was applied to cases, cure of clinical and subclinical mastitis was better. Thus only adding one of the evaluated tests to the treatment strategy is not enough to enable prudent antimicrobial usage. Apparently, farmers use predictive information to determine the current mastitis treatment strategy, which could not be substituted by a result of the tests used. Given the added value of diagnostic information in the mastitis treatment strategy, combining diagnostic information with (cow related) predictive information, for example in decisions trees, might be a step towards further enhancing prudent antimicrobial use for mastitis on Dutch dairy farms.

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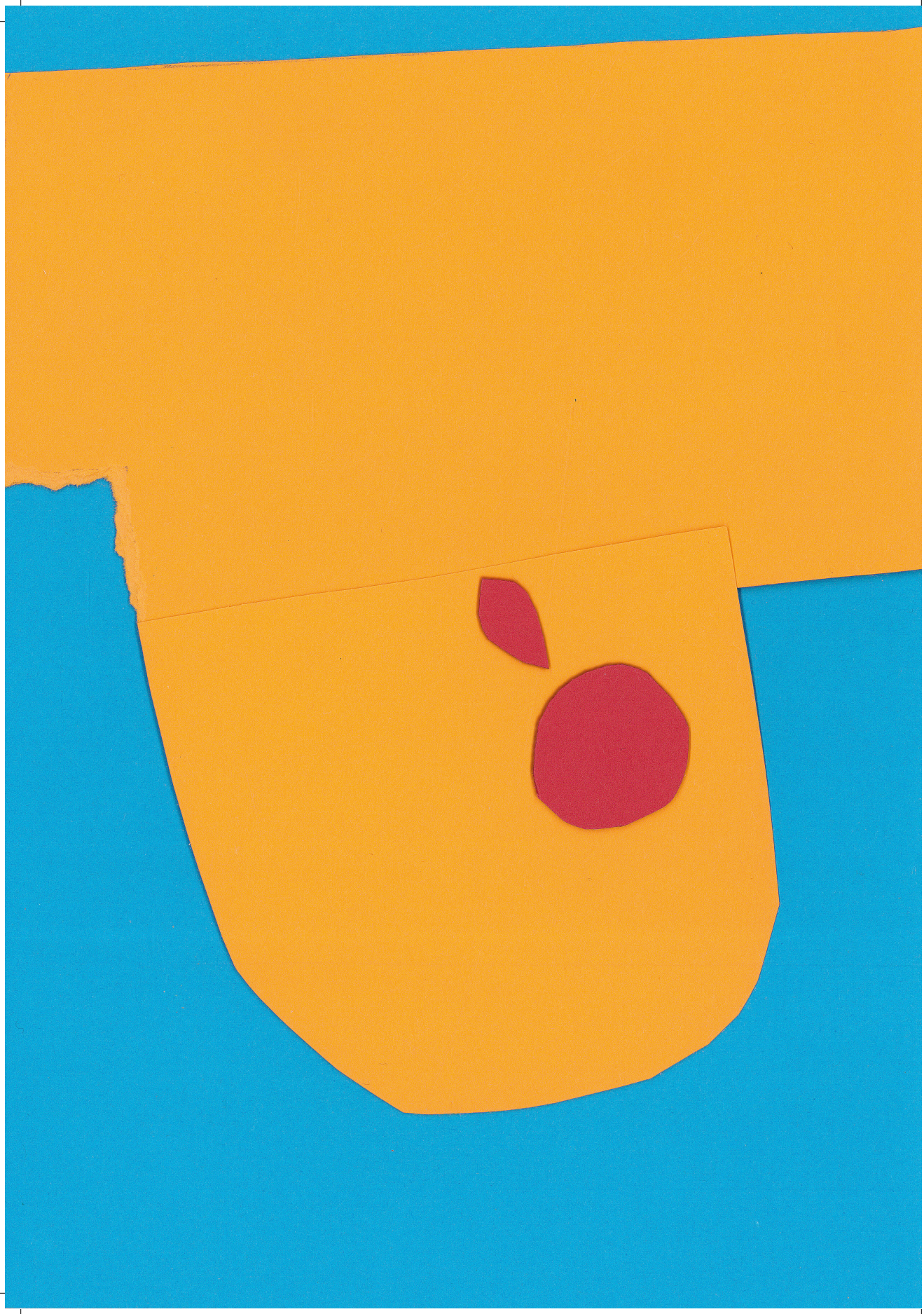

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Samenvatting

Het aantal testen dat buiten een laboratorium kan worden uitgevoerd, bijvoorbeeld naast de patiënt, groeit. De toename in deze zogenaamde *point-of-care* (POC) testen is zowel in humane als in veterinaire geneeskunde te zien. Een van de redenen daarvoor is de kortere tijd tussen monstername en testuitslag, wat een snellere diagnose mogelijk maakt en daarmee kan helpen om de ziekte gericht te behandelen.

Mastitis (uierontsteking) is de ziekte die het grootste aandeel heeft in het gebruik van antimicrobiële middelen op melkveebedrijven. Door het gericht gebruik van antimicrobiële middelen wordt de kans op antimicrobiële resistentie-ontwikkeling in bacteriën verkleind. Het gebruik van mastitistesten kan ervoor zorgen dat de meeste antimicrobiële middelen gebruikt op melkveebedrijven gericht worden ingezet, iets wat in andere landen al is aangetoond. Ondanks dat testen beschikbaar zijn om het prudent gebruik van antimicrobiële middelen te ondersteunen, worden ze weinig gebruikt door Nederlandse veehouders in behandelbeslissingen voor mastitis. Aangezien veehouders vaak twijfelen over behandelbeslissingen voor mastitis, hebben Nederlandse melkveehouders mogelijk behoefte aan additionele informatie over de oorzaak om zo de beslissing te vergemakkelijken en om prudent gebruik van antimicrobiële middelen voor mastitis te stimuleren.

Het doel van dit proefschrift was het bepalen van de toegevoegde waarde van diagnostische informatie voor de mastitisbehandelstrategie van Nederlandse melkveehouders. Het ultieme doel is om bij te dragen aan verbeterde mastitisbehandelingen op melkveehouderijen om zo het prudent gebruik van antimicrobiële middelen te stimuleren.

Nederlandse melkveehouders lijken behoefte te hebben aan betrouwbare diagnostische mastitistesten, zoals ze hebben aangegeven tijdens de telefonische enquête die beschreven is in *hoofdstuk 2*. Voor klinische mastitis zijn veehouders vooral geïnteresseerd in een test die adviseert over het te kiezen antimicrobiële middel, terwijl ze voor subklinische mastitis meer geïnteresseerd zijn in een test die de oorzakelijke bacterie aangeeft. Twee keer zoveel veehouders zouden een test gebruiken voor behandelbeslissingen voor klinische en subklinische mastitis, en bij droogzetten, wanneer zo’n test binnen acht tot 24 uur een uitslag geeft en beschikbaar is voor gebruik op het bedrijf.

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In *hoofdstuk 3* zijn de resultaten van een laboratoriumevaluatie beschreven van vier op kweek gebaseerde commercieel verkrijgbare testen die gebruikt kunnen worden op het melkveebedrijf. Deze testen zijn vergeleken op hun mogelijkheid om mastitispathogenen aan te tonen in melkmonsters die routinematig naar een laboratorium zijn gestuurd. Alle vier de testen zijn ongeveer even goed in staat om Gram-positieve bacteriën, Gram-negatieve bacteriën en geen groei aan te tonen. Daarom kunnen alle vier de testen gebruik worden in de behandelstrategie voor mastitis, zonder dat een van de vier testen kan worden aangewezen als de beste test.

Op kweek gebaseerde testen hebben een tijd-tot-resultaat van 24 uur, wat een nadeel kan zijn wanneer deze testen voor behandelbeslissingen worden gebruikt. In *hoofdstuk 4* wordt de ontwikkeling en evaluatie beschreven van vier *loop-mediated isothermal amplifiction* (LAMP) assays, die een korte tijd-tot-resultaat hebben. De assays om *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, en *Streptococcus* spp. aan te tonen, zijn gebruikt op isolaten en veldmonsters. Ook is een *nucleic acid lateral flow immunoassay* (NALFIA) geëvalueerd als een eenvoudige uitleesmogelijkheid. De overeenkomst van de assays ten opzichte van de referentie methode was bijna perfect (≥ 0.80), behalve voor de *Streptococcus* spp. assay, die een lagere overeenkomst liet zien. De assays kunnen veelbelovend zijn voor snelle on-site diagnostiek om mastitis-veroorzakende pathogenen aan te tonen op voorwaarde dat een eenvoudige en snelle isolatie van DNA beschikbaar komt.

In *hoofdstuk 5* zijn de resultaten beschreven van een telefonische enquête en een veldstudie naar de toegevoegde waarde van diagnostische informatie in de droogzetstrategie van veehouders. In de enquête gaven Nederlandse melkveehouders aan behoefte te hebben aan additionele testen bij droogzetten. Ook hebben ze interesse in behandelbeslissingen op kwartierniveau, als stap naar een verdere reductie in het gebruik van antimicrobiële middelen. Tijdens de veldstudie ontvingen veehouders diagnostische informatie op kwartierniveau, naast de informatie die ze al beschikbaar hadden bij droogzetten. Desondanks werden behandelbeslissingen op koeniveau genomen. Het beschikbaar stellen van diagnostische informatie zorgde ervoor dat antimicrobiële middelen vaker werden gebruikt dan wanneer veehouders hun huidige methode toepasten. Het aantal gerichte behandelingen nam wel toe. Het selectief gebruik van diagnostische informatie voor koeien met hoog celgetal bij droogzetten kan resulteren in de meest optimale aanpak om prudent gebruik van antimicrobiële middelen bij droogzetten te stimuleren.

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In *hoofdstuk 6* worden de resultaten beschreven van een veldstudie waarin veehouders twee op kweek gebaseerde testen gebruikten om te beslissen over de behandeling van klinische en subklinische mastitis. De behandelstrategie met gebruik van een test is vergeleken met de huidige behandelstrategie zonder test op de mate van genezing van de koe en de mate van gebruik van antimicrobiële middelen. In geval een test werd gebruikt in de behandelstrategie werden meer behandelingen gericht ingezet dan wanneer de huidige strategie werd gevolgd, zowel voor klinische mastitis als voor subklinische mastitis. De mate van gebruik van antimicrobiële middelen was gelijk of lager wanneer een test werd gebruikt in de behandelstrategie voor klinische mastitis, maar hoger bij subklinische mastitis. De mate van genezing was gelijk of lager wanneer een test werd gebruikt in de behandelstrategie voor klinische en subklinische mastitis dan wanneer de veehouder de huidige behandelstrategie volgde. Dit suggereert dat veehouders aanvullende waarnemingen meewegen in hun beslissingen, informatie die niet meegenomen wordt in het resultaat van de geëvalueerde testen.

Er bestaat geen perfecte referentietest om de oorzaak van mastitis aan te tonen. Daarom wordt in *hoofdstuk 7* de evaluatie van twee op kweek gebaseerde testen door middel van *latent class analysis* beschreven. Op beide testen zijn mastitisveldmonsters gekweekt zowel in het laboratorium, als door veehouders op het bedrijf. Drie categorieën zijn geëvalueerd: de aanwezigheid van Gram-positieve bacteriën, van Gram-negatieve bacteriën, en geen groei. Eén test was minder sensitief in het aantonen van Gram-positieve bacteriën wanneer deze op het bedrijf werd gebruikt. Over het algemeen waren de testen uitgevoerd in het laboratorium beter in staat de drie categorieën aan te tonen dan de testen uitgevoerd op het bedrijf.

In *hoofdstuk 8* worden de belangrijkste resultaten en conclusies van dit proefschrift bediscussieerd. Veehouders staan positief tegenover het prudent gebruik van antimicrobiële middelen voor de behandeling van mastitis. Zij beschouwen diagnostische informatie als van toegevoegde waarde in de behandelstrategie voor klinische mastitis. On-farm testen kunnen diagnostische informatie leveren om mastitisgevallen te categoriseren in een van de drie diagnostische categorieën (Gram-positieve bacteriën, Gram-negatieve bacteriën of geen groei). Hiermee kunnen behandelbeslissingen voor mastitis geoptimaliseerd worden voor wat betreft het gericht toepassen van antimicrobiële middelen tegen aangetoonde vermoedelijk mastitis-veroorzakende pathogenen. Aangezien de huidige behandelstrategie van veehouders een betere genezing van klinische en subklinische mastitis liet zien dan wanneer een test werd

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gebruikt in de behandelstrategie, lijkt enkel het toevoegen van een van de geëvalueerde testen niet voldoende om antimicrobiële middelen prudent te gebruiken. Kennelijk gebruiken veehouders voorspellende informatie om de huidige mastitisbehandeling te bepalen. Gegeven de toegevoegde waarde van diagnostische informatie in de behandelstrategie voor mastitis, lijkt het combineren van deze informatie met voorspellende (koegebonden) informatie, in bijvoorbeeld beslisbomen, de volgende stap richting het vergemakkelijken van het prudent gebruik van antimicrobiële middelen voor mastitis op Nederlandse melkveehouderijen.

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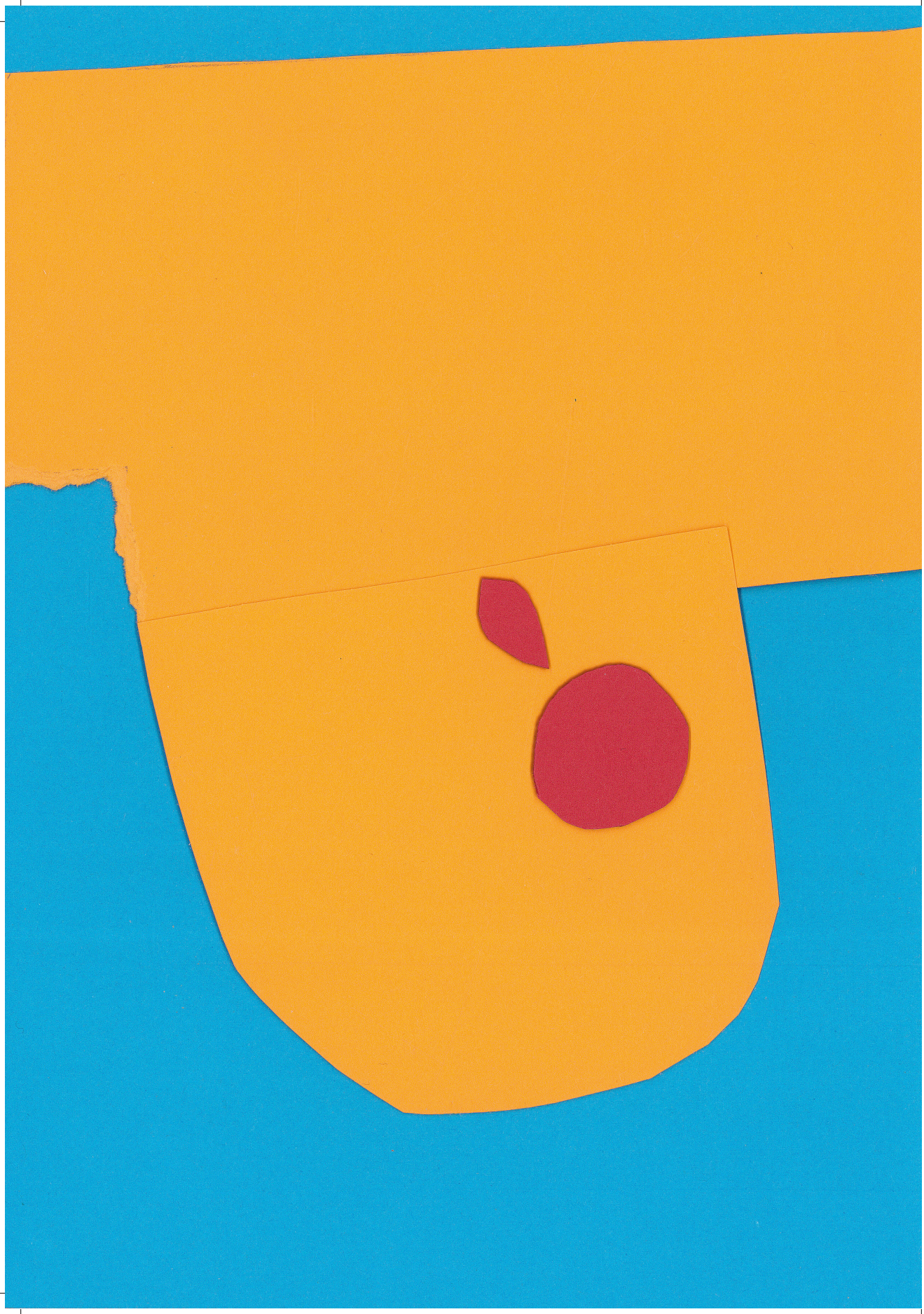

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About the author

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Karien Griffioen was born on 2 February 1984 in Hollandsche Rading (Maartensdijk, the Netherlands). During secondary school she did an internship at the faculty of Veterinary Medicine, Utrecht University, where she participated in a study after bovine ovaria. From their her interest in conducting research was born. She finished 'het Gemeentelijk Gymnasium' in Hilversum in 2002 and started to study veterinary medicine at Utrecht University. She graduated in 2010 and worked for a few years as a dairy cow practitioner in Overijssel en Flevoland. During this work, she was often questioned by farmers how to treat a cow with mastitis. When the opportunity came, she returned to 'Utrecht' in 2014 where she started her PhD to study the added value of diagnostic information for mastitis treatment decisions. The results of this research are presented in this thesis. During her PhD, Karien followed the postgraduate master Epidemiology at Utrecht University and graduated in 2019 with a specialization in Veterinary Epidemiology. Currently, Karien is working as project manager in cattle research at Royal GD, Deventer, the Netherlands.

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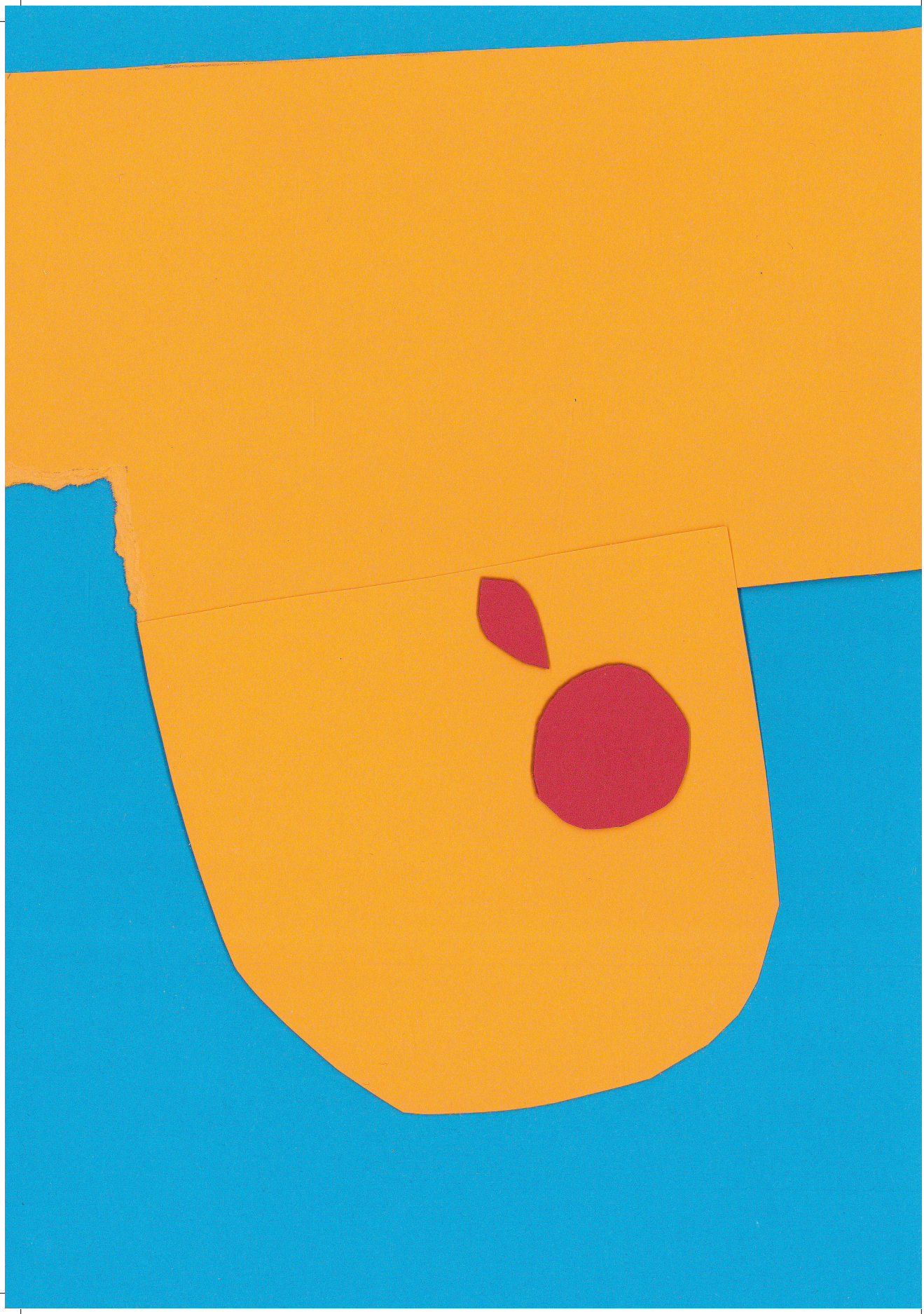

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