ON-FARM STRATEGIES FOR THE PREVENTION AND DETECTION OF GRAM-SPECIFIC CLINICAL MASTITIS IN DAIRY COWS

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ACADEMIC ABSTRACT

Controlling mastitis in dairy herds relies on good prevention and detection methods. This dissertation describes two areas of research relating to mastitis control. In the first objective, the efficacy of 2 vaccines against *Escherichia coli* mastitis in mid-lactation dairy cows was evaluated. Secondly, in a series of 3 studies, milk and activity sensor data were used to derive models for clinical mastitis (CM) detection, and models were tested for their ability to indicate the causative pathogen type.

Primiparous and multiparous animals were vaccinated with 1 of 2 commercially available J5 vaccines (V1 or V2) or served as unvaccinated controls (CTL). Intramammary challenge with *E. coli* approximately 84 d later resulted in few treatment differences in the clinical and behavioral responses, except that vaccinated cows exhibited fever (≥ 39.4 °C) 3 h earlier and laid down for longer periods than CTL. Although vaccinated cows had similar severity and duration of CM, V1 cows produced more serum IgG1 and IgG2 than V2 cows. Our results indicated that the effects of vaccination were diminished in mid-lactation, and that antibodies are not the limiting factor in defending against induced *E. coli* mastitis.

Multiple regression models, incorporating the slope changes in relevant milk and activity sensor data, were developed to indicate all CM cases (ACM), or specifically, CM due to Gram-negative (GN) or Gram-positive (GP) bacteria. Gram-specific models had greater detection accuracy (> 80%) than the ACM model (75%) when evaluated using the model training dataset, but independent evaluation demonstrated reduced sensitivity (Se) of detecting CM by all models (GN, 62%, ACM, 56%, and GP, 32% Se). Data in the 3 d prior to CM were more important in detecting GN pathogens, whereas the best GP models incorporated changes more than 1 week prior to CM detection. Still, model performance was imperfect. Next, models were rederived from a dataset that better reflected the infection distribution of the herds its use was intended for. However, the Se of detecting CM in real-time, across 2 farms, was < 21% for all models, and categorization by Gram-status had no benefit. An insufficient number of CM cases was considered to contribute to the poor detection performance of models and limited repeatability across farms. Consequently, models derived in this study were inadequate for implementation as mastitis detection tools. In the future, development of new sensors and application of more sophisticated algorithms to the field of mastitis detection may improve the accuracy of models using sensor data.

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GENERAL ABSTRACT

Mastitis is an important disease of dairy cattle that adversely affects animal welfare, productivity, and milk quality. Controlling mastitis in dairy herds relies on good prevention and detection methods. In this dissertation, we investigated two elements of mastitis control: 1) the effects of vaccination in protecting against mastitis, and 2) the ability of on-farm sensor data to detect clinical mastitis (CM) and indicate the causative pathogen type.

Coliform bacteria commonly cause CM, and vaccination against these bacteria can reduce the severity of the disease. We evaluated the effect of 2 different vaccines on the clinical, behavioral, and immune response in cows with experimental mastitis caused by *Escherichia coli*. Our findings indicated that the effects of vaccination had diminished at the time of experimental mastitis, as vaccinated cows had no improvement in clinical recovery compared with unvaccinated controls. Although no clinical or behavioral differences were observed between the 2 different vaccines, the antibody response differed, suggesting that antibodies are not the key player underpinning the mechanisms of vaccination against induced coliform mastitis in mid-lactation.

Rapid detection and diagnosis of mastitis is important to reduce effects on the cow, and to support decision making for the appropriate intervention. We aimed to develop and test mastitis detection models that utilized data collected by on-farm sensor technologies. Milk and activity parameters, which may be differentially affected by mastitis depending on the pathogen causing infection, were used in multiple regression models for detecting any CM case, or specifically CM caused by Gram-positive or Gram-negative bacteria. Gram-specific models were initially estimated to have > 80% accuracy in classifying cows with and without mastitis, but further validation demonstrated that the models were not repeatable when tested independently. Subsequently, models that were more suited to the farms they were to be implemented on were developed, and tested, revealing limited performance in detecting any case of CM, or CM due to the Gram-specific pathogens. Model derivation was limited by an insufficient number of CM cases to represent the variation in different cases of CM within the Gram-positive and Gram-positive classifications. Although our models did not show promise as a mastitis detection tool, milk and activity data may be incorporated with other sensor data for improved detection and diagnosis of mastitis.

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Introduction

Mastitis is defined as inflammation of the mammary gland, predominantly in response to pathogenic bacteria, with adverse effects on animal welfare and productivity, milk quality and farm profitability (Barkema et al., 2009). Mastitis occurs in clinical and subclinical forms. Clinical mastitis **(CM)** is identified by the abnormal appearance of milk, such as clots, flakes or discoloration in milk, or by changes to the udder, including swelling, redness, or increased temperature of the infected gland. For any case of CM, the infection can be classified as mild, moderate or severe, based on the clinical presentation of the disease. Mild and moderate CM exhibit milk and mammary gland abnormalities, whereas severe cases have systemic involvement, and can lead to death (Pinzón-Sánchez et al., 2011). Subclinical mastitis (**SCM**), unlike CM, is not visibly detectable, but involves inflammation that is indicated by indirect measures, such as an elevated somatic cell count **(SCC)** in the milk.

Mastitis continues to be a common disease of dairy cows; an estimated 25% of cows are affected by CM annually in the US (USDA, 2016). The average cost of a case of CM was estimated as $326 (Liang et al., 2017), and up to $444 for a CM case occurring in the first 30 days of lactation (Rollin et al., 2015). The economic impact of mastitis includes direct costs for treatments, veterinary attention, discarded milk, labor, and temporary or permanent loss in milk production, with the latter representing more than two thirds of the total losses incurred by mastitis (Akers and Nickerson, 2011). Indirect costs include SCC penalties, missed premiums for milk quality rewards, premature culling and cow replacement, and reduced reproductive performance (Santos et al., 2004).

Producers have a responsibility to produce high quality milk, with low SCC and bacterial counts. In accordance with the Food and Drug Administration's Grade A Pasteurized Milk

Ordinance, abnormal milk is to be discarded, and bulk tank SCC **(BTSCC)** must not exceed 750,000 cells/mL (FDA, 2015), though many states and processors have introduced SCC limits below the federal limit (APHIS, 2016). For international export, the SCC limit is 400,000 cells/mL (Hillerton and Berry, 2004). In addition, the use of antimicrobial products in livestock production systems is under increasing scrutiny, and mastitis, being a major contributor to antibiotic use on dairy farms, has been identified as an area where reductions can be made (van Werven, 2013). To achieve these milk quality requirements and prudent use of antimicrobials, attention to mastitis control measures is necessary, specifically mastitis prevention and detection.

The purpose of this literature review is to outline mastitis etiology and pathophysiology, summarize mastitis prevention strategies with a specific focus on vaccination against coliform mastitis, and discuss methods for detecting and diagnosing mastitis, including those that incorporate precision technologies adopted on-farm. This literature review will culminate in the research objectives that provide the focus for this dissertation.

Etiology and pathophysiology of mastitis

Etiology of mastitis

Mastitis and intramammary infection **(IMI)** are terms that are often used interchangeably, but it is important that the differences are recognized. Although the definitions of CM and SCM do not require the presence of an etiological agent, most cases are caused by a pathogenic microorganism that induce the inflammatory response known as mastitis. The term IMI infers the presence of a microorganism in the milk, but does not stipulate whether inflammation is involved (Berry and Meaney, 2006). Therefore, the diagnosis of mastitis involves measuring the inflammatory response, whereas the diagnosis of IMI involves determining the etiological agent (Adkins and Middleton, 2018).

The predominant cause of mastitis is bacteria, and most cases are due to one primary pathogen (Watts and Yancey, 1994). More than 140 different species have been reported as the cause of a case of bovine mastitis (Watts, 1988), but approximately 10 bacterial species or groups of species account for more than 95% of mastitis infections (Makovec and Ruegg, 2003; Bradley et al., 2007). Bacteria invade the mammary gland, usually entering via the teat canal, and if bacterial multiplication rates surpass recognition by the host immune system, an infection is established, triggering the inflammatory response. Though less common, non-bacterial etiological agents of bovine mastitis include yeast and *Prototheca* spp. (Watts, 1988).

Mastitis-causing pathogens can be split into various categories, depending on source and transmission of bacteria, severity of infection, or pathogenesis within the host. The primary reservoir of bacteria, and how those bacteria are contracted and transferred between animals, can enable categorization of mastitis pathogens as contagious or environmental (Bramley et al., 1996).

Contagious pathogens, as the name suggests, tend to spread between cows in the milking parlor, through contact with contaminated milk in the milking equipment or on milkers hands (Neave et al., 1969). These pathogens usually originate within an infected mammary gland, because a host is required for bacteria to survive and propagate. The pathogens most commonly considered to spread contagiously are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp, but infections caused by other pathogens can be spread via contagious means (Zadoks et al., 2001; Klaas and Zadoks, 2018). *Staphylococcus aureus* is a frequent isolate from mastitis cases in many countries, whereas the prevalence of *Strep. agalactiae* and *Mycoplasma* spp. is much lower in countries with a developed dairy industry, but can still be associated with herd outbreaks (Keefe, 1997; Fox, 2012).

Environmental pathogens tend to inhabit the cows' surroundings, such as fecal material, bedding, contaminated water, and on body sites of the cow, and do not require a host to survive (Hogan and Smith, 2003). Cows are most susceptible to infection by environmental pathogens in the period between milkings. Many species of environmental bacteria can cause mastitis, but those frequently responsible include several species of streptococci (other than *Strep. agalactiae*), such as *Streptococcus uberis* and *Streptococcus dysgalactiae*, *Enterococcus* spp., and Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*, also known as coliforms (Klaas and Zadoks, 2018).

Mastitis pathogens can also be classified as major or minor, reflecting the severity of infection. Major pathogens such as *Staph. aureus*, streptococci spp., and coliforms cause more damage to mammary tissue, indicated by greater increases in SCC and likelihood of causing CM. Minor pathogens include coagulase-negative staphylococci **(CNS)** and *Corynebacterium bovis*, and are typically associated with only small or moderate increases in SCC (Harmon, 1994; Oliver and Calvinho, 1995). Minor pathogens are often described as opportunistic, living within the environment and taking advantage of the host. However, *C. bovis* can behave as a contagious pathogen (Watts, 1988), but is easily controlled by application of teat disinfectants.

Classifying pathogens by the way in which the host recognizes the invading pathogen and generates an immune response, splits many mastitis-causing bacteria into Gram-positive and Gram-negative pathogens. The distinction is made by Gram staining, which differentially stains bacterial cell walls based on the peptidoglycan content. For Gram-positive bacteria, peptidoglycan makes up between 50 to 90% of the cell wall, whereas Gram-negative bacteria have less peptidoglycan, encased within other cellular layers or membranes, and that represents only 10% of the cell wall (Beveridge, 2001). Gram-negative and Gram-positive bacteria contain different pathogen-associated molecular patterns **(PAMPs)** that are recognized by the host immune system, specifically by pattern recognition receptors **(PRR)**. The Toll-like

receptor **(TLR)** family in mammalian hosts are PRR; TLR-4 recognizes lipopolysaccharide **(LPS)** of Gram-negative bacteria and TLR-2 recognizes lipoteichoic acid **(LTA)** of Gram positive bacteria (Ginsburg, 2002). Interactions with different TLR initiates distinct immune responses for Gram-negative and Gram-positive bacteria, which provides an opportunity for Gram-specific mastitis detection and control measures. The distinction of Gram-negative and Gram-positive bacteria is a common theme of this dissertation, and will be continually referred back to.

Evolution of mastitis control

The diversity of pathogens causing mastitis has increased over decades of mastitis research, concurrent with changing systems and herd management practices to handle larger herd sizes (reviewed by Ruegg, 2017a). Traditionally, when control programs were established in the 1960's, the contagious pathogens, *Strep. agalactiae* and *Staph. aureus*, were most prevalent. Control measures, referred to as the 5-point plan, included 1) the use of a post-milking teat disinfectant, 2) use of dry cow antibiotics in all quarters of all cows, 3) appropriate treatment of CM cases, 4) culling chronically infected cows, and 5) maintenance of milking equipment (Neave et al., 1969). The 5-point plan was largely successful in reducing the prevalence of contagious pathogens, especially *Strep. agalactiae*, by promoting the widespread use of dry cow therapy, and teat disinfection.

In the late 1970's, mastitis due to environmental bacteria was becoming more common, as the control measures against contagious pathogens were less effective in limiting environmental IMI (Smith et al., 1985). Although bulk tank SCC could be maintained at satisfactory levels, the rates of CM were still high in herds with contagious pathogens under control. For environmental bacteria, 81% of coliform IMI and 53% of environmental streptococci IMI detected at calving progressed to CM during that lactation (Smith et al., 1985). Mastitis incidence was also closely linked with populations of bacteria in bedding for streptococci

(Smith et al., 1985) and coliforms (Hogan et al., 1989). Two additional control points were proposed by Smith et al. (1985); first, reduce teat end exposure through cleaner environments and bedding, and second, increase resistance of cows to infection, with immunization identified as one approach.

More recently, the issue of antimicrobial use in livestock production systems and the resulting societal pressure has led to reassessment of mastitis control recommendations. Administration of dry cow antibiotics to all quarters of all cows has been a recommended practice since the 1960's (Neave et al., 1969), but a selective approach to dry cow therapy is now advised. This selective approach requires elevated SCC or confirmed presence of IMI to select cows for treatment, and is mandatory in some European countries (van Werven, 2013). Moreover, treatments of CM are usually administered at the time of detection, prior to knowing the bacteriological status of the affected quarter. The shift in the predominant cause of mastitis from contagious to environmental pathogens increased the proportion of cases due to Gram-negative pathogens, and cases of CM with no detectable pathogen in culture, neither of which are suitable candidates for broad-spectrum antibiotic treatment. Therefore, non-specific treatment of CM leads to unnecessary use of antimicrobials. Methods to detect CM, and diagnose the causative pathogen or pathogen type helps to direct appropriate treatments or interventions, with responsible antimicrobial stewardship in mind.

Impacts of mastitis on milk yield and composition

The changes in milk constituents observed during a case of mastitis differ by pathogen type, and the severity and location of infection within the mammary gland. In a healthy lactating mammary gland, tight junctions are impermeable, providing a barrier function to restrict paracellular movement between the alveolar lumen and interstitial space (Linzell and Peaker, 1972). Ion-pumping systems maintain ion concentrations on either side of the apical membrane of epithelial cells (Kitchen et al., 1980). The resident population of immune cells in the

mammary gland, typically < 100,000 cells/mL, are crucial in determining the severity and duration of infection (Sordillo and Streicher, 2002). During mastitis, inflammatory processes and bacterial products damage mammary epithelial cells at the site of infection, resulting in increased permeability of tight junctions and disturbance of membrane potentials, allowing luminal and interstitial components to move down their concentration gradients (Linzell and Peaker, 1972).

More detail regarding the interaction between the host and the pathogen will follow in a subsequent section, but first the impacts of mastitis on the host will be considered. In general, mastitis lead to reductions in milk yield, and milk components such as lactose, casein, and milk fat, whereas blood-based proteins, ions and somatic cells increase (Kitchen, 1981). The changes are more drastic for major pathogens such as *Staph. aureus*, streptococci and coliforms, compared with minor pathogens.

Milk yield is primarily affected by the reduced number and synthetic capacity of secretory cells at the site of infection (Seegers et al., 2003). Lactose is also a major influencer of milk yield, as it is the main osmotic regulator of milk volume. Changes in lactose during mastitis have been well-documented (Kitchen, 1981; Auldist et al., 1995; Pyörälä, 2003). Lactose declines due to reduced synthesis by mammary epithelial cells, and leakage into interstitial fluid (Kitchen, 1981). Additionally, some bacteria (e.g., coliforms) ferment lactose as a source of energy. As lactose moves into the interstitial space, water follows by osmosis, reducing the amount of milk in the alveolar lumen, and depressing the total milk yield. Reductions in milk yield of between 0 and 9.5% of the total produced per lactation (0 to 350 kg) have been reported (reviewed by Hortet and Seegers, 1998). Major pathogens, especially *E. coli*, are typically associated with greater extent of decline in milk production, whereas the effects of infection by minor pathogens on milk yield are rarely noticeable.

The change in fat content of mastitic milk has been debated by different authors (reviewed by Kitchen, 1981). Triglycerides, which are the main form of milk fat, are broken down by bacterial lipases. Thus, an increase in free fatty acids and short chain fatty acids may be observed (Kitchen, 1981). It was thought that fat synthesis was only slightly depressed during mastitis (Bruckmaier et al., 2004), but the associated decline in milk yield may increase the percentage of fat in the milk. Over the lactation, total milk fat yield will be less due to the decrease in milk production as a result of mastitis.

There is little change in the total protein content of milk; however, the proportions of specific proteins change dramatically. Milk proteins decline, whilst serum proteins increase due to tight junction leakiness. The overall change in protein percent depends on the degree of damage sustained by the mammary tissue (Auldist et al., 1995). The concentration of casein, the primary milk protein, was 12% less than in non-mastitic milk (Harmon, 1994), explained by reduced synthesis, and degradation by bacterial proteinases. Reduced synthetic activity and leakage via paracellular pathways can also explain the reduction of whey proteins, α-lactalbumin and β-lactoglobulin, in milk. Conversely, blood proteins such as serum albumin and plasmin move through the blood-milk barrier in large quantities. Tissue damage can be detected through monitoring enzymes lactate dehydrogenase (**LDH**) and N-acetyl-B-D-glucosaminidase (**NAGase**) in milk. Lysis of host cells releases LDH, a stable cytoplasmic enzyme. Phagocytic actions release the hydrolytic lysosomal enzyme, NAGase, which correlate closely with SCC (Kitchen, 1981).

The milk SCC increases due to the recruitment of immune cells, predominantly neutrophils, and may remain elevated for some time following bacteriological clearance of the infection (Oliver and Calvinho, 1995). Epithelial cells that slough off during tissue damage can also contribute to the SCC in milk. The SCC rapidly increases in response to *E. coli* infection and

rapidly regresses, whereas the SCC response to *Strep. uberis* is delayed and for *Staph. aureus* can fluctuate widely due to the evasiveness of this pathogen (Schukken et al., 2011).

Changes in ion concentrations in the milk include an increase in sodium and chloride, and reduction in potassium, calcium and citrate. The failure of the tight junctions to maintain the concentration gradient set up by the Na-K-ATP pump on the apical surface of the epithelial cells allows flow via the paracellular pathway, which is inaccessible in a normal lactating gland (Linzell and Peaker, 1972). Calcium is associated with casein in milk so reduced casein concentrations contributes to reduced calcium (Harmon, 1994). Many of the changes in milk composition have been utilized in cow-side tools for indicting CM, and these will be discussed in more detail in a later section.

Impacts of mastitis on cow behavior

In general, diseases in mammalian species involve behaviors that conserve energy, because the immune response against invading pathogens is energetically costly (e.g., fever; Johnson, 2002). However, because dairy cows are prey animals, they are prone to covering up sickness behavior (Leslie and Petersson-Wolfe, 2012). Nonetheless, mastitis is a painful condition, and cows will display changes in behavior in accordance with the physiological changes and pain experienced, which is associated with the severity of mastitis (Dantzer and Kelley, 2007).

A large study established that healthy cows housed in free-stalls spent 11 ± 2.1 h lying/d (660 min/d), broken up into 9 ± 3 bouts/d of 88 ± 30 min/bout in duration (Ito et al., 2009). Whilst variation exists between farms and between individual cows (Ito et al., 2009), cows experiencing mastitis either naturally or experimentally, spent less time lying, compared with clinically healthy control cows (Cyples et al., 2012; Fogsgaard et al., 2015). In experimental challenge studies, reduced time lying was particularly evident in the early stages of an acute infection, such as that caused by infusion of live *E. coli* (Yeiser et al., 2012) or LPS (Siivonen et al., 2011). Likewise, Yeiser (2011) reported divergent lying behavior between healthy cows

and cows that went on to develop CM naturally 1 to 2 d later. The local effects of mastitis including udder swelling and increased intramammary pressure might discourage cows from lying around the time of CM detection.

Because cows with mastitis typically spend less time lying, more time will be spent either walking or standing idle. Due to the nature of CM, reports on behaviors prior to CM are limited, but are increasing with the greater adoption of precision technologies. Cows were less active from 5 d before diagnosis of naturally occurring *E. coli* CM (Stangaferro et al., 2016) or 3 d before diagnosis of CM due to any pathogen (King et al., 2018), compared with healthy cows. Deviations in activity in response to Gram-negative CM were also more pronounced compared with Gram-positive CM (Stangaferro et al., 2016). Gram-positive bacteria such as *Staph. aureus* can cause a chronic infection, where changes in behavior are less likely to occur (King et al., 2018).

Inconsistent behaviors have been reported after CM has been detected in cows that received an intramammary challenge of *E. coli* or LPS. Step activity was greater following infusion of LPS (Siivonen et al., 2011), whereas the time spent standing idle was also increased upon CM detection in cows challenged with *E. coli* (Fogsgaard et al., 2012). The variability in activity and behavior of individual cows suggests that using a cow as her own baseline will have more value in identifying deviations associated with conditions such as mastitis (Ito et al., 2009; King et al., 2018).

Changes in feeding behavior in response to disease have been described in natural infections and challenged cows (Fogsgaard et al., 2015; Gonzalez et al., 2008). In the days prior to CM detection, feed intake was less in mastitic cows, compared with healthy controls (Fogsgaard et al., 2015), which has been attributed to reduced rate of eating, and fewer visits and less competitive behavior at the feed bunk (Sepulveda-Varas et al., 2016). After experimental

intramammary challenge with *E. coli*, feed intake and rumination time was less (Yeiser et al., 2012; Fogsgaard et al., 2012), and time spent eating was reduced (Zimov et al., 2011). The decline in feed intake was proposed to contribute to the decline in milk yield observed in cows experiencing mastitis (Yeiser et al., 2012).

Host response to bacterial infection

The ability of a cow to prevent bacteria from establishing an infection within the mammary gland depends on a multitude of factors, but these broadly involve the innate, or natural, anatomical and biochemical defense mechanisms, which prevent pathogens from entering and infecting the gland; the efficiency of the host immune system to recognize, immobilize and remove pathogens that gain entry; and the ability of the pathogen, and its virulence factors, to evade these defense mechanisms.

Innate immune defenses

The first line of defense to prevent bacterial entry involves innate anatomical barriers, such as the teat skin, streak canal, teat sphincter muscle, and keratin plug. Together, these maintain a tightly closed barrier between milkings, and help to trap and physically obstruct bacteria from passing through the streak canal into the mammary gland. Antimicrobial compounds, such as cationic proteins and long-chain fatty acids, have been isolated from keratin, with bacteriostatic or bactericidal effects (Sordillo and Streicher, 2002). Streptococci and some coliforms are inhibited by the antimicrobial components in keratin. If bacteria evade these anatomical barriers, they can adhere to internal tissues, multiply and release intracellular products within teat and gland cisterns. Bacteria may then establish an infection, and progress up the ductal network into smaller ducts and alveolar areas (Zhao and Lacasse, 2008).

In the early stages of infection, innate immunity provides the predominant response, mediated by soluble factors, such as complement and lactoferrin, and a cellular response. Complement has an important role in enhancing phagocytes and antibody activity, but is in low

concentrations in the milk of uninfected glands. Lactoferrin, as an iron-binding protein, has inhibitory effects on coliforms, as they require iron for growth (Hogan and Smith, 2003).

The cellular response aims to identify and destroy the foreign agent, and initiate inflammation. To recognize a vast array of microorganisms known for causing infection in the bovine mammary gland, the innate system, through PRR, discriminates between foreign and self-molecules in a non-specific manner by recognizing PAMP (Bannerman, 2006). Cells that are positioned to come in contact with invading pathogens, such as resident macrophages and epithelial cells, express PRR (Bannerman, 2006). The most notable PRR is the TLR family; TLR-2 recognizes Gram-positive peptidoglycan and LTA, and TLR-4 recognizes LPS from Gram-negative bacteria. Lipoteichoic acid acts as an adhesion molecule for Gram-positive bacteria, facilitating bacterial colonization within the host. Upon lysis of bacterial cells, LTA are released, stimulating inflammation (reviewed by Ginsburg, 2002). Gram-negative bacteria appear not to adhere to tissue, and instead can multiply in secretion (Opdebeeck et al., 1988). Lipopolysaccharide of Gram-negative bacterial cell walls are made up of 3 components: an outer polysaccharide chain (O antigen), an inner core of polysaccharides, and a lipid A phospholipid layer. Lipid A is the portion responsible for eliciting toxic effects of fever and shock, and is only exposed upon cell death. The basis for vaccines against coliforms is the exposure of the inner core of polysaccharides, as the O antigen is absent in the rough mutant strain of *E. coli* (J5) commonly used in vaccine development (Tyler et al., 1990).

Activation of TLR receptors by LTA or LPS produces an intracellular signaling cascade, which initiates the inflammatory response (Sharma and Jeong, 2013). Resident macrophages and epithelial cells are stimulated to produce chemotactic factors for immune cell recruitment, and stimulates secretion of various pro-inflammatory mediators including cytokines, prostaglandins, and leukotrienes (Oliver and Calvinho, 1995). Cytokines involved in the early response include Interleukin-1 (**IL-1**), IL-6, and tumor necrosis factor α (**TNF- α**). Neutrophil

migration to the infection site is assisted by adhesion molecules on the surface of endothelial cells. During the periparturient period, chemotactic neutrophil recruitment may be impaired due to the down-regulation of the cellular adhesion receptors (Kehrli and Shuster, 1994). The production of pro-inflammatory mediators facilitates vasodilation, for increased blood flow to the mammary gland to expedite immune cell recruitment, and increased vascular permeability, which assists movement of immune cells into affected mammary tissue, under primary stimulation by prostaglandins (Oliver and Calvinho, 1995). The inflammatory processes accommodate movement of fluid into tissues, causing pain, edema, and increased mammary pressure, with effects on cow behavior. Different mediators are released in response to different pathogen types, and the combinations released will determine extent of input by the adaptive immune system (Bannerman, 2006).

Once neutrophils have been recruited, they become the principal cell type, contributing to more than 90% of the total leukocyte population (> 10^6 cells/mL; Persson et al., 1993). Neutrophils act by engulfing foreign or infected cells by phagocytosis, or by destruction of bacteria in an oxygen-dependent or oxygen-independent manner, often causing collateral damage to nearby host cells. *Staph. aureus* have the ability to avoid phagocytosis and survive inside neutrophils, which is one mechanism behind the chronic infection that they are known to cause. Neutrophil activity becomes more effective with the presence of opsonic antibodies (Sordillo and Streicher, 2002). The production of such antibodies against specific pathogens is associated with adaptive immunity, and will only be triggered if the pathogen manages to evade the innate defenses.

Adaptive immune defenses

The adaptive, or acquired, immune response provides longer-lasting protection against the specific bacterial antigen that elicited a response, and consists of cell-mediated and humoral immunity. The cell-mediated response is facilitated by the interaction between T lymphocytes

and antigen presenting cells (e.g., macrophages, B lymphocytes). Following phagocytosis of bacteria, macrophages (as an antigen presenting cell) digest and present the bacterial antigen on the cell surface, in conjunction with major histocompatibility complex (**MHC**) molecules, in a process called antigen presentation (Sordillo and Streicher, 2002). Naïve T cells respond to antigen presentation by differentiating into mature T cells with receptors that will recognize only one antigen. Two T lymphocytes subsets CD4+ (T-helper (**Th**) cells) and CD8+ (cytotoxic T (**Tc**) cells) play a pivotal role in implementing cell-mediated immunity against extracellular and intracellular bacteria, respectively. T-helper cells stimulate cytokine production and T and B lymphocyte clonal expansion to initiate humoral immunity, whereas Tc cells defend against pathogens once inside a host cell, in association with MHC Class I receptors. Activation of T cells requires the presence of Interferon-γ (**IFN-γ**) and IL-2, two of the major pro-inflammatory cytokines. At parturition however, production of these cytokines may be reduced, which may account for the suppression of the normal immune function at this time (Sordillo and Streicher, 2002).

Humoral immunity relates to antibody production by B lymphocytes, which recognizes specific antigen. Activation of B cells is achieved through interaction with Th cells and MHC receptors as before, but the secretion of IL-2 prompts B cell differentiation into plasma cells. Each plasma cell is responsible for producing one unique antibody type, and these can provide immunological memory and thus a stronger, quicker immune response if the same pathogen is subsequently encountered, referred to as an amnestic response (Burton and Erskine, 2003). The antigen specificity and the amnestic response provide the foundations for vaccination as a strategy to enhance immunity (Erskine, 2012).

Antibodies are important in opsonization and alerting phagocytic cells to destroy bacteria and infected cells. Different classes of antibodies (immunoglobulins (**Ig**)) exist, each with different roles in immune defenses. IgM is the primitive Ig, with other classes undergoing class-

switching based on the immune signals received. A primary function of IgM is as an opsonin to activate the complement cascade, an innate defense system. IgM may be more effective as blood proteins infiltrate the alveolar lumen during mastitis. IgA is present in milk and can neutralize bacterial toxins. IgA can also deter bacterial adherence through agglutination, helping to facilitate bacterial removal with the flow of milk through ducts (Sordillo and Streicher, 2002). The IgG antibody class are more abundant in colostrum, have been reported to disturb neutrophil function in early lactation by down-regulating or blocking the antibody receptor on neutrophils, preventing binding of opsonized pathogens (Burton and Erskine, 2003). During inflammation, IgG subtypes play an important role in opsonization. The IgG2 subtype is a more effective opsonin than IgG1; low abundance of serum IgG2 was associated with increased mastitis incidence (Mallard et al., 1998). The efficacy of antibodies during different stages of lactation, or during inflammation, is proposed to be altered as one of the mechanisms of vaccination.

The interaction between the innate and adaptive immune system, maintained by cytokines and other chemical messengers, is vital to ensure bacteria are detected and killed in a timely manner, while ensuring a rapid regression of the inflammatory response to minimize damage to mammary tissue.

Mastitis prevention

Prevention of mastitis is a critical element of mastitis control on dairy farms, since prevention is more desirable than cure. Preventative approaches to mastitis rely on those that reduce bacterial exposure at the teat ends, or enhance mammary gland immunity. Reducing bacterial exposure requires maintenance of a clean, dry environment, from the milking parlor, through laneways, and to the bedding. Hygienic preparation of teats prior to milking is a common pre-milking procedure in the US, involving application of a teat dip to reduce bacterial load on teats prior to milking (Blowey and Collis, 1992). Teat end exposure can be further reduced by

applying a post-milking teat disinfectant, and by offering feed immediately, to prevent cows from laying during the time when the streak canal remains open (Barkema et al., 1999). Good milking machine function ensures optimum vacuum stability and reduces the risk of overmilking, which minimizes teat end damage and reduces opportunities for bacteria to colonize teat ends. Regular maintenance and replacement of bedding will help manage bacterial counts in bedding material, minimizing bacterial contamination of teat ends.

Strategies that optimize mammary gland immunity include vaccination, dietary supplements, immunomodulators, and breeding for enhanced resistance to mastitis. Although these approaches may not entirely prevent mastitis, they can influence the risk of new IMI, or the severity of CM, both of which are beneficial outcomes for cows and producers. For this review, the role of vaccination in enhancing mammary defenses against mastitis will be considered, specifically focusing on J5 vaccines against coliform bacteria.

The goals of vaccination are generally to reduce new IMI, eliminate existing infections, or reduce the incidence and severity of CM (Yancey, 1993); however, it is unlikely that one vaccine will achieve all of these expectations. The greatest challenge for developing effective mastitis vaccines is the heterogeneity in mastitis pathogens (Klaas and Zadoks, 2018). Commercially available mastitis vaccines target mainly coliforms and *Staph. aureus*, though vaccines against the latter are still not considered satisfactory (Landin et al., 2015).

Vaccination for coliform mastitis

The severity of infections due to coliform bacteria can range from an acute, mild or moderate case with short-term impacts on milk production and milk quality, and a relatively quick recovery, to a severe, systemic illness, which can lead to death. In general, approximately 25% of cows in a well-managed herd develop clinical mastitis caused by coliform bacteria, with most cases occurring in early lactation (Hogan and Smith, 2003). As such, vaccination of dairy

cattle with a J5 bacterin has been a successful approach to reduce the severity of coliform mastitis for the past 30 years (Gonzalez et al., 1989). In the US, an estimated 18% of all dairy operations and 51% of large dairy herds (> 500 cows) have adopted a vaccination program against coliform mastitis (USDA, 2016).

The strain often incorporated in coliform vaccines is *E. coli* O111:B4 (**J5**), a rough mutant that lacks the O-antigen of LPS, exposing the inner core of polysaccharide as the antigen (Tyler et al., 1990). Immunization with a J5 vaccine produces cross-reactive antibodies with other Gram-negative pathogens, providing non-specific immunity, because the target is a highly conserved region of LPS (Dosogne et al, 2002; Chaiyotwittayakun et al., 2004). Commercially available J5 vaccines recommend different immunization protocols, but commonly 2 to 3 doses are administered subcutaneously, with the first dose given at dry off, a second dose 2 to 3 wk before calving, and a third dose may be give within the first 2 wk of lactation. Considerable research has gone into developing J5 vaccines, including the adjuvant incorporated to enhance the response to antigen (e.g., Hogan et al., 2005), the route of administration (e.g., Tomita et al., 1998), dosage, and the number and timing of vaccine doses (e.g., Chaiyotwittayakun et al., 2004; Erskine et al., 2007).

Field studies evaluating J5 vaccine efficacy have demonstrated reduced incidence and severity of CM in vaccinated cows, and elevated antibody levels compared with controls, but indifferent rates of IMI at calving. Cows immunized with a J5 vaccine had reduced incidence of naturally occurring CM caused by Gram-negative bacteria in the first 3 mo of lactation, compared with unvaccinated controls (Gonzalez et al., 1989; Cullor, 1991; Hogan et al., 1992). However, no controlled field study has demonstrated reduced risk of Gram-negative IMI in vaccinates at calving, suggesting that vaccination does not play a role in preventing or eliminating infection (Hogan and Smith, 2003). Nevertheless, antibody response has been influenced by J5 vaccination; the production of J5-specific IgG1 and IgG2 was greater in vaccinates

immediately after calving (28 d post-vaccination), but the difference was no longer evident following CM (Wilson et al., 2009). Furthermore, vaccinated cows have shown smaller reductions in milk yield, and a reduced likelihood of premature culling due to CM, compared with unvaccinated controls (Wilson et al., 2009).

Experimental models of *E. coli* mastitis have demonstrated reduced severity and duration of CM following mild (Hogan et al., 1995) and moderate experimental *E. coli* mastitis (Tomita et al., 1998; Hogan et al., 2005). Although vaccinated cows had greater peak bacterial counts following intramammary *E. coli* challenge, a faster reduction was observed, and shorter duration of IMI (80 h), compared with unvaccinated controls (130 h; Hogan et al., 1995). In a later study, vaccinates had reduced bacterial counts and SCC, and a shorter duration of abnormal milk appearance than unvaccinated controls, following induced *E. coli* mastitis (Hogan et al., 2005). Similarly, a faster reduction in *E. coli* concentration in milk and quicker milk yield recovery was reported in vaccinates, whereas vaccination did not affect the duration of clinical signs (Tomita et al., 1998). In contrast, some studies have shown few clinical differences following *E. coli* intramammary challenge, but an antibody response was associated with J5 vaccination (Smith et al., 1999; Tomita et al., 2000). Contradictory results are not surprising in *E. coli* intramammary challenge studies, with potential explanations including: differences in vaccines and adjuvants used (Hogan et al., 2005), stage of lactation and time since last vaccination (Wilson et al., 2009), and differences in concentration and virulence of the bacterial strain being infused at challenge (Smith et al., 1999; Vangroenweghe et al., 2004).

The classical hypothesis for the mechanism underpinning J5 vaccination is that increased antibody titers to *E. coli* J5 in vaccinated cows enhances opsonization of LPS, thereby neutralizing antigen, and flagging it for efficient clearance by phagocytes (Tyler et al., 1991; Hogan et al., 1992). Possibly, the reduced bacterial numbers contribute to less severe signs of

CM (Hogan and Smith, 2003). Additionally, a role for cell-mediated immunity in enhancing protection by J5 vaccination has been proposed (Schukken et al., 2011).

An influx of neutrophils to the mammary gland occurs on recognition of LPS, and the speed of neutrophil mobilization is an important factor in host defense against *E. coli* (Hill et al., 1983; Burvenich et al., 2003). Neutrophil trafficking and activation is mediated by T cells, emphasizing the link between innate and cell-mediated immunity (Shafer-Weaver et al., 1999). The immune response in early lactation favors a T-helper type 2 (**Th-2**) response, with greater production of IgG1, whereas later in lactation, the response shifts to T-helper type 1 (**Th-1**), with increased IgG2 production, which is more protective against mastitis (Shafer-Weaver et al., 1999). The Th-1 cells promote inflammation through cytokine production, which initiate class switching from IgM to IgG2. An important function of IgG2 is as an opsonin that facilitates phagocytosis by neutrophils (Stevens et al., 1988; Estes and Brown, 2002). Vaccination with J5 is hypothesized to reverse the Th-2 response common in early lactation, to a Th-1 pro-inflammatory state (Schukken et al., 2011).

Most experimental challenge studies induce mastitis during the first month of lactation to evaluate vaccination efficacy. Few research studies have considered the effects of vaccination on mastitis occurring later in lactation, when coliform bacteria can still cause severe illness. Antibody levels are known to decline over time, hence the need for successive vaccinations with the start of each lactation (Wilson et al., 2009). One field study demonstrated a diminished effect of J5 vaccination as lactation progressed and time since the last vaccination increased (Wilson et al., 2009). In early lactation, vaccinated cows had a lesser decline in milk production following CM than controls, but the advantage was no longer observed after 75 days in milk (**DIM**; Wilson et al., 2009). Gram-negative bacterins elicit a weak amnestic response for IgG1 and IgG2 (Kehrli and Harp, 2001), offering a probable explanation for the short duration of protection conferred by J5 vaccination (Erskine, 2012).

The value of vaccination on CM after peak lactation has received little research attention. Hyperimmunization (6 doses) of cows reduced the incidence of severe CM cases between 42 and 126 DIM, compared with cows that received 3 doses (Erskine et al., 2007), and greater IgG1 and IgG2 titers were established in hyperimmunized cows in a later study (Erskine et al., 2010). Although 2 to 3 doses are routine in industry, additional doses may help to protect against coliform mastitis later in lactation. Whether additional doses prove to be cost effective is unknown, but would vary substantially between herds (Wilson et al., 2009).

Mastitis detection

Although mastitis prevention should take precedence, mastitis will always be an issue as long as cows and bacteria coexist, so response strategies are necessary for when disease occurs. Early detection of mastitis can enable earlier intervention and potentially a better response to treatment (Milner et al., 1997), reducing the severity of disease and the risk of infection spreading to other cows in the herd. In this section, common methods for mastitis detection on conventional farms will be described, leading to the use of precision, sensor-based technologies for CM detection, how mastitis detection systems are evaluated, and the current performance of these sensor-based systems.

Detection of mastitis on conventional farms

Detection of CM on conventional farms relies on visual assessment of milk and the mammary gland for abnormalities during pre-milking teat preparation. For mild cases of CM, the milk appears visibly abnormal with clots, flakes or discoloration, whereas in moderate cases of CM, the mammary gland also shows involvement such as swelling, redness or increased temperature. Severe CM is a systemic condition, encompassing the signs of moderate CM, with fever, reduced appetite, dehydration and diarrhea apparent in affected cows (Hogan and Smith, 2003). Typically, more than 85% of CM cases present as mild or moderate cases (Oliveira et al., 2013).

Subclinical mastitis differs from CM, in that the milk and mammary gland appear visibly normal, even though inflammation is occurring (Harmon, 1994). As a result, subclinical mastitis is detected by indirect methods, most commonly by individual cow SCC, and the California mastitis test (**CMT**) on conventional farms. The SCC indicates the number of somatic cells (primarily leucocytes) per milliliter of milk, and on conventional farms, individual cow SCC is measured through regular (i.e., monthly) Dairy Herd Improvement Association (**DHIA**) testing. Thresholds commonly used to indicate infection are usually in the range of 100,000 to 200,000 cells/mL (Dohoo and Leslie, 1991; Sargeant et al., 2001).

The CMT is a cow-side tool for indicating subclinical mastitis, whereby a reagent is added to milk to lyse somatic cells, releasing DNA which precipitates in the solution. The viscosity observed is roughly proportional to the SCC (Schalm and Noorlander, 1957). The scoring system and SCC approximation for each score have been reported as: N = negative (65,000 cells/mL), T = trace (114,000 cells/mL), 1 = weak positive (394,000 cells/mL), 2 = distinct positive (1,700,000 cells/mL), and 3 = strong positive (7,000,000 cells/mL; Luedecke et al., 1967), with a score of T and above indicating infection. The CMT offers an inexpensive, easy-to-use tool, but its interpretation can be subjective.

Detection of mastitis using precision technologies

Technological advances and applications within biological systems, along with increasing herd sizes and the shortage of a suitable farm workforce, has led to the rise in popularity of robotic milking (Penry, 2018), also known as automated milking systems **(AMS)**. The detection of mastitis in AMS differs to that in conventional systems, because farm staff are no longer involved in the milk harvesting process when visual assessment of milk usually occurs. Because the milk quality and CM detection requirements remain the same as for conventional systems, an alternative method of detecting CM for mastitis control in AMS is necessary.

Mastitis detection has been automated using precision technologies and algorithms that process data to alert producers to cows suspected of having mastitis (Hogeveen and Ouweltjes, 2003). Sensors used in detecting mastitis are attached or non-attached (i.e., not wearable), with the non-attached sensors categorized further as inline or online. Inline sensors continuously measure milk from the beginning of milk flow to the end of milking, whereas online sensors sample a small amount of milk to measure the component of interest (Penry, 2018). Inbuilt sensors measure electrical conductivity, SCC (or a proxy), milk color, udder temperature, milk yield, or hydrolytic or proteolytic enzymes, or a combination of these measures, to detect mastitis (Hogeveen et al., 2010).

The electrical conductivity describes the resistance of milk to an electric current, and indicates mastitis by the increased concentration of sodium and chloride ions in milk from infected cows (Hogeveen et al., 2010). Since the 1960's, conductivity has been used in mastitis detection, firstly by hand-held meters, and later, through inline sensors (Rutten et al., 2013). In milk from uninfected quarters, the electrical conductivity typically ranges between 4.0 and 5.0 mS/cm (Hamann and Zecconi, 1998). Estimated conductivity in abnormal milk varies widely between individual animals, herd and with the severity of infection; therefore, the threshold to determine an infection varies. Often, the conductivity of mastitic milk exceeds 6.0 mS/cm, though a lower threshold might be appropriate for detecting subclinical mastitis (Sheldrake and Hoare, 1981; Norberg et al., 2004). Conductivity can also be affected by factors other than mastitis, including temperature, milk fat content and the milk fraction assessed (Nielen et al., 1992). Infected quarters have greater conductivity at the beginning and end of milking; consequently, mean values from infected quarters show greater variation across milking, whereas healthy quarters have more stable conductivity values across a milking (Norberg et al., 2004). Interquartile range of conductivity values at a single milking may be preferred over an absolute threshold due to the variation in individual cows (Nielen et al., 1992).

Most sensors measuring the SCC of milk are online, taking a small sample during milking to perform analyses. Elevated SCC is associated with greater probability of CM (Steeneveld et al., 2008), due to the role of immune cells, especially neutrophils, in defending against pathogens causing IMI. Commercially available sensors for online SCC measurement either stain and optically count cells using flow cytometry (e.g., Løvendahl and Sørensen, 2016), or automate the CMT to indirectly estimate the SCC by assessing milk viscosity (Whyte et al., 2005). As for electrical conductivity, variation exists in the SCC threshold used to indicate an infection, but is commonly between 200,000 and 300,000 cells/mL (Schukken et al., 2003; Dufour et al., 2011). Not surprisingly, estimates using quarter level SCC are more valuable in detecting mastitis than cow composite SCC, and allow the opportunity for within cow comparison at a single milking (Mollenhorst et al., 2010).

Sensors measuring milk color were thought to be more indicative of CM, because they relate to visible changes rather than indirect measures of inflammation, such as SCC or conductivity (Hogeveen et al., 2010). On-line color sensors use a light emitting diode to measure the differences in light reflected from milk at red, green and blue wavelengths (Espada and Vijverberg, 2002). Milk, being a white substance, typically reflects light equally between the different wavelengths, but when milk color changes due to abnormalities associated with mastitis, the light reflected at each different wavelength will not be equal. Milk from quarters with CM had lower color values than normal milk (Espada and Vijverberg, 2002). Most applications of sensors measuring color are not useful alone, but can add value in combination with SCC or electrical conductivity (Hogeveen et al., 2010).

An increase in udder temperature due to inflammation is a sign of CM, and may have some application in detecting severe CM. Infrared thermography applications in humans demonstrated that radiated heat emitted from the skin can be measured (Jones and Plassmann, 2002). In bovine applications, a predictive model for udder surface temperature based on

repeated measures of ambient temperature and healthy cows showed some promise in mastitis detection (Berry et al., 2003). In an experimental mastitis model, temperature changes at the udder were detected by thermal imaging, but authors concluded that temperature would not provide an early indicator of mastitis (Hovinen et al., 2008). Like color sensors, temperature may only be useful when combined with other mastitis indicators (Maatje et al., 1997).

Milk yield declines due to mastitis, but the magnitude depends on severity of infection and the type of pathogen causing infection, as well as the cow's parity and stage of lactation (Gröhn et al., 2004). Milk yield serves as a non-specific indicator of mastitis, because decreased milk production can occur for many reasons (e.g., diet changes, estrus, metabolic diseases, lameness, heat stress), but value may lie in its use in combination with other parameters related to mastitis (Jensen et al., 2016; Kamphuis et al., 2010). Sensors are also capable of measuring milk lactose, protein and fat percentages inline, and because mastitis has an impact on milk composition, the changes may be indicative of mastitis. The only component with some application in indicating IMI is lactose (Berning and Shook, 1992; Hamann and Krömker, 1997). Lactose percent declines in response to mastitis, whereas changes in protein and fat percent are not as clear-cut. Like other milk parameters measured, consideration of changes in components over time, or in comparison to other quarters at the same time, are expected to provide more predictive power.

Some commercially available mastitis detection tools are able to measure mastitic enzymes online, including LDH, a cytoplasmic enzyme from host cells that is released upon cell lysis (Kitchen, 1981). Studies show a strong association between LDH and SCC in mastitic cows (Chagunda et al., 2006). An increase in LDH occurs prior to detection of CM, illustrating the predictive potential of this measure, but stage of lactation can affect LDH levels; concentrations are higher at the beginning and end of lactation (Chagunda et al., 2006).

Pedometers are primarily used for estrus detection, but recognition that behavioral changes prior to disease detection has identified an additional purpose for technologies measuring step activity, lying time and position changes (Siivonen et al., 2011; Stangaferro et al., 2016). To date, no mastitis detection system uses activity sensor data for mastitis detection. Individually, activity measures are not expected to have value in indicating mastitis, but there is potential for their application in combination with other parameters (Petersson-Wolfe et al., 2017).

Evaluating mastitis detection systems

A mastitis detection system consists of one or more sensor devices that measure the parameter(s) of interest, and the software that collects and processes data to generate information or an alert (Rutten et al., 2013). In the case of mastitis, the information or alert generated indicates a cow suspected of having mastitis, at a single milking or day. The performance of mastitis detection systems, or a test designed to detect disease, can be described by the terms, sensitivity (**Se**) and specificity (**Sp**; Saah and Hoover, 1997). Sensitivity represents the ability of the system to correctly identify a true case of mastitis, whereas Sp describes the system's ability to correctly identify non-mastitic animals. False negative results occur when the system's result is negative, but mastitis is truly occurring (IDF, 2011). False positives occur when the system incorrectly identifies disease, in a non-diseased cow (IDF, 2011). Sensitivity and specificity are inversely proportional, because a change in test characteristics will generally be reflected by an increase in one and reduction in the other.

To a non-scientific audience, Se and Sp, as performance indicators, can be confusing. Therefore, the terms success rate (**SR**) and false alert rate per 1000 milkings (**FAR1000**), were introduced to describe system performance for detecting CM within a practical farm setting (Hogeveen et al., 2010). The SR is the proportion of alerts that are correct, which is synonymous with the positive predictive value **(PPV).** The FAR1000 depicts the number of

false alerts that occur in 1000 cow milkings, emphasizing to producers the frequency of alerts that, when checked, prove to be a false alarm.

Standardized evaluation of mastitis detection systems is difficult due to the variation in the gold standard used, and the time window within which an alert is a true positive (Kamphuis et al., 2016). A cow with mastitis is usually the gold standard for evaluation of mastitis detection systems; however, whether that is a cow with an IMI detected in culture, a cow with an elevated SCC, or a cow with CM detected by visibly abnormal milk, must be clearly defined. Furthermore, the period used to describe an alert as successful (when the alert occurs concurrently with the CM event) differs between studies. Time windows of up to 17 d have been reported (de Mol et al., 1997), and as the time window increases, model performance usually increases, because there is a greater chance of an alert coinciding with the CM event (Hogeveen et al., 2010).

An ideal mastitis detection system would alert true cases of mastitis in a timely manner, while producing very few false alerts (Hogeveen et al., 2010). Although early detection (before clinical signs) of a CM case is desired, if too much time passes between an alert and the CM event, the producer may perceive the alert as false and lose trust in the system (Hogeveen et al., 2010). The recommended time window is 5 milkings (usually 2.5 d; Kamphuis et al., 2016). Whether the goals of the mastitis detection system are to limit the number of missed cases (high Se) or reduce the number of false alerts (high Sp), depends on the farm using that system, but optimized systems would have combined high Se and Sp. According to the ISO/FDIS 20966 for AMS, a mastitis detection should reach 99% Sp at a set Se of 80% (Hogeveen et al., 2010).

Performance of current mastitis detection systems

Previous research has illustrated that no single measure, or sensor, can perfectly indicate mastitis. Mastitis detection using CMT was limited in performance, with Se around 70% and

Sp between 50% and 70% (Sanford et al., 2006; Roy et al., 2009). Though it has limitations, the CMT still provides value as a screening tool on dairies. The relationship between SCC and mastitis is well documented, but the ability of SCC to accurately indicate IMI depends on the number and frequency of SCC measures available (Reneau, 1986). Previously, monthly SCC estimates for individual cows were not sufficient, but with larger herd sizes and more automation led to the development of systems for automated mastitis detection.

Detection models have evolved over time from univariate models (Sheldrake and Hoare, 1981), often using electrical conductivity, to multivariate models that combine milk yield, temperature, color or SCC to improve detection accuracy (Nielen et al., 1995; Kamphuis et al., 2008). Further, more sophisticated algorithms using neural networks (Nielen et al., 1995), time series combined with Kalman filters (de Mol et al., 1997; Chagunda et al., 2006), fuzzy logic (Cavero et al., 2006), decision tree induction (Kamphuis et al., 2010), discriminant analysis (e.g., Mottram et al., 2007), and naïve Bayesian networks (Steeneveld et al., 2010a; Jensen et al., 2016) have been applied in CM detection models. Reviewing the different modeling approaches used is beyond the scope of this review.

The performance of current mastitis detection systems for identifying CM has been reviewed extensively (Hogeveen et al., 2010; Rutten et al., 2013). From the 37 sensor systems evaluated for automated mastitis detection, the reported Se ranged from 55% to 89%, and Sp from 56% to 99% (Rutten et al., 2013). Few mastitis detection systems had high combined Se and Sp, and some of those that did were limited by sample size, or used an impractically wide time window (reviewed by Rutten et al., 2013).

Improvements in sensor performance have been described when multiple sensors are used in combination (e.g. electrical conductivity with SCC; Mollenhorst et al., 2010). One study reported high Se (85%) and Sp (99%) when combining quarter measures of milk color,

conductivity, quarter yield ratio, and the time taken for milk flow to commence. However, the success is cautioned, because only 26 CM cases were used to develop and validate the model, and the study was not peer reviewed (Song and van der Tol, 2010). A recent study utilized a greater variety of sensor inputs than many previous studies, including milk composition and non-sensor information in a multi-variate, dynamic linear model in combination with a naïve Bayesian classifier to produce a probability of mastitis (Jensen et al., 2016). Using this approach, the estimated Sp was 81%, at a Se set at 80%. The Bayesian approach has been identified to easily handle missing observations, which are common in sensor technology (Steeneveld et al., 2010a; Jensen et al., 2016). The addition of non-sensor information such as DIM, parity, and CM history can also increase detection accuracy, reducing the number of false positives by 35% in one study (Steeneveld et al., 2010a). Improvements may also be made by using quarter based measures (as for AMS), enabling comparisons within a cow at a single milking, or comparing changes over time that are more sensitive (Claycomb et al., 2009; Hogeveen et al., 2010).

Although a range of sensor systems for indicating mastitis exist, a shortage of validated detection systems providing information to support decisions has been identified (Rutten et al., 2013). Without validation and decision support, herdsmen are presented with sensor data and must interpret and make decisions, which results in much subjectivity and variation between individuals. Additionally, authors agree that these systems are useful, especially in AMS where some automated tool for detection CM is necessary; however, improvements are necessary to reach the desired Se of 80% and Sp of 99% (Hogeveen et al., 2010). A Sp below 99% will result in many false alerts, but some producers may accept lower levels of Sp to create attention lists as a screening tool to assist mastitis detection (Kamphuis et al., 2008). Improvements can be made in the sensors themselves, or in the algorithms that process the sensor data. The false alert rate is a common complaint of mastitis detection systems, and researchers have

investigated methods to rank alerts, usually in association with severity of infection (Steeneveld et al., 2010b).

Diagnosis of intramammary infection

The identification of a bacterial pathogen from an aseptically collected milk sample is the definitive diagnosis of an IMI, first proposed by the International Dairy Federation (**IDF**) in 1975 (IDF, 2011). Diagnosing the etiological agent supports treatment decisions and emphasizes areas where mastitis control can be improved, based on the predominant pathogen type being isolated.

Laboratory methods

The traditional diagnostic test for identifying pathogens in milk is bacterial culture, with the presence of bacteria indicated by growth on an appropriate medium following an incubation period (Middleton et al., 2017). Though most bacteria readily grow on generic blood-based media under aerobic conditions at 37 °C, some pathogens require specialist growth conditions (e.g., *Mycoplasma* spp). Bacterial species are identified based on phenotypic characteristics, indicated by Gram staining, assessing colony morphology, and serotyping and analyzing enzymatic profiles. No method can definitively determine the 'true' bacteriological status of a mammary gland, but culture procedures were generally accepted as the only method to reliably detect the cause of an IMI (Hogan et al., 1999; Dohoo et al., 2011).

Over the past decade, molecular technologies have offered alternative or confirmation tests for pathogen diagnosis from milk. Multiplex, real-time polymerase chain reaction (**PCR**) detects DNA sequences that are unique to a species or group of bacteria, thus confirming the presence or absence of bacterial DNA. In less than 4 hr, a diagnosis can be made, providing a faster, and potentially more sensitive alternative to bacterial culture (Koskinen et al., 2010). However, the application of PCR in mastitis diagnostics still has pitfalls, compared with culture, and is

associated with greater costs (Adkins and Middleton, 2018). Another laboratory method with relatively novel application in mastitis diagnostics is loop-mediated isothermal amplification (**LAMP**), which also amplifies DNA, and has a short time-to-result (Bosward et al., 2016). Matrix Assisted Laser Desorption/Ionization Time of Flight (**MALDI-ToF**) mass spectroscopy is a secondary method for pathogen diagnosis, offering a high throughput, rapid approach that uses a protein fingerprint in comparison to a reference database (Adkins and Middleton, 2018). This approach is no quicker than culture, because it usually cannot detect the pathogen directly from milk, unless the bacterial concentrations are sufficiently high; therefore, this test requires growth of the bacteria in culture first.

On-farm diagnostic tools

Most producers do not routinely collect milk samples for pathogen diagnosis via laboratory analysis, but tools have been developed to allow for on-farm pathogen diagnosis to encourage more targeted CM treatment (Adkins and Middleton, 2018). On-farm culture (**OFC**) systems essentially bring the laboratory to the farm, in that the principles of bacterial culture are applied at the farm, with simplified interpretation, as diagnosis is not at the species level. Biplate and triplate systems have been developed, which use a combination of media for selective growth of Gram-negative bacteria, streptococci or staphylococci, on MacConkey, TKT and Baird-Parker agar, respectively (Royster et al., 2014; McCarron et al., 2009). The time to a diagnosis is less than 1 d, which is quicker than sending a sample to a culture laboratory (Royster et al., 2014). Interpretation has been simplified so that producers differentiate by pathogen type (Gram-negative, Gram-positive and no pathogen isolated) to inform treatment decisions. However, OFC systems were regarded as cost-effective only in herds with Gram-negative pathogens as the dominant cause of CM (Down et al., 2017). Recently, application of PCR techniques has become possible on-farm (Acumen Detection, 2018), reducing the time-to-result to half a day, but the investment and running costs are expected to be greater than OFC.

Treatment protocols based on intramammary infection diagnosis

The decision to treat CM is often made without knowledge of the quarter's bacteriological status, because no cow-side diagnostic tool is available to rapidly identify the etiological agent. For most producers, a CM case is treated upon detection using a broad-spectrum antimicrobial (Oliveira et al., 2013), but not all CM cases warrant antibiotic treatment. Factors that should be considered when deciding on treatment of a cow with CM include: the etiological agent, the CM history of the cow, and knowledge of therapeutic principles to enable selection of an appropriate, approved antimicrobial product (Ruegg, 2017b).

The rates of spontaneous cure and the assumed efficacy of antibiotics were considered when evaluating the need for antimicrobial treatment of CM due to specific pathogens (Ruegg, 2018). Depending on the selected antibiotic, treatment was assumed to be most efficacious for CM due to environmental streptococci (80 to 95%), and CNS (40 to 80%), as only a small proportion of environmental streptococci (28 to 30%) and about half of CNS cases were expected to spontaneously cure (Ruegg, 2018). Less than 50% of coliforms and cases with no pathogen isolated were assumed to benefit from treatment with a highly efficacious antibiotic, as spontaneous cure rates were high for *E. coli* (80 to 95% of cases) and moderate for *Klebsiella* spp. (25% to 60%). The distribution of pathogens causing CM varies between herds and countries, but weighted averages collated from various studies suggests that 26% of CM cases had no pathogen isolated in culture, 26% were caused by coliforms, 22% by environmental streptococci, 8% by CNS, 12% by *Staph. aureus* and 6% of CM cases were due to other pathogens (Ruegg, 2018). Consequently, the overall proportion of CM cases thought to benefit from antimicrobial treatment ranged from 20% to 33% (Ruegg, 2018). Therefore, in agreement with previous estimates (Roberson, 2012), up to 80% of antibiotics used in the treatment of non-severe CM are not justified (Ruegg, 2018).

Due to the recognized need for judicious antimicrobial use, and the knowledge that not all CM cases benefit from antibiotics, pathogen-specific treatment protocols for mild and moderate CM have been advocated (Suojala et al., 2013; Roberson, 2012). The benefits of pathogen identification were demonstrated in a series of studies by Lago et al. (2011a, 2011b). Selective treatment based on OFC systems provided significant reductions in discarded milk, and more than halved the antimicrobial use, compared with the 'treat all with clinical signs' approach (Lago et al., 2011a). Basic treatment advice for the selective group was to treat Gram-positive infections, and leave Gram-negative and 'no growth' infections. Even though treatment for cows in the selective group was delayed (1 to 2 d), no significant difference in clinical or bacteriological cure rates, SCC, milk yield or lactational survival was demonstrated between the two groups (Lago et al., 2011b). Although these treatment recommendations have been worthwhile in mild and moderate CM cases, for severe CM, emergency veterinary attention and appropriate treatments are recommended, because prioritization of animal welfare is paramount (Suojala et al., 2013; Ruegg, 2018).

Although OFC systems have been successful, implementation on all farms is not practical. Adoption is more likely on large farms, and users must be competent in culturing and interpreting samples, including growth from contaminants. Consequently, an alternative method for diagnosing the etiological agent accurately and rapidly is needed. Such information would support prudent antimicrobial use, while enabling selection of an appropriate intervention to optimize cure (Lago and Godden, 2018).

Potential for sensor data to indicate the pathogen type

The differences in pathogenesis of mastitis due to Gram-negative and Gram-positive bacteria outlined support the need for Gram-specific control measures that inform treatment decisions. The availability of an enormous amount of data, collected daily for each cow in the herd,

provides a valuable opportunity to address this need. Could sensor data differentiate CM cases caused by Gram-negative and Gram-positive bacteria?

Few have attempted to categorize CM cases by Gram-status using sensor data (Kamphuis et al., 2011; Steeneveld et al., 2009). Steeneveld and colleagues (2009) used a naïve Bayesian network to develop probability distributions for the Gram-status of 1,202 CM cases, with 73% accuracy. Inputs into the naïve Bayesian network included Gram-specific CM history, milk color, SCC, and whether the cow was sick or not. A smaller study of 140 CM cases described a decision tree incorporating various electrical conductivity and milk color measurements at the quarter level (Kamphuis et al., 2011). For predicting the Gram-status, the accuracy was 91%, based on the training dataset, but accuracy dropped to 55% for a test dataset. Authors suggested a need for more CM cases for building the decision tree, or the integration of more or improved sensor and non-sensor information. Further categorization of Gram-positive pathogens has been proposed to produce greater improvements in detection performance, but so far, the number of cases has been a limiting factor (Steeneveld et al., 2009; Kamphuis et al., 2011).

An automated approach to Gram-specific CM identification could be used on a large scale and, if accurate, would provide a better opportunity to inform treatment decisions. We propose that improvements upon current Gram-specific models can be made by using more sensor variables, especially those that capture the different time series changes in milk composition and cow behavior that occur prior to CM diagnosis for infections due to Gram-negative or Gram-positive bacteria.

Research objectives

Mastitis is an issue on all dairy farms and control measures should place greater emphasis on prevention, and early detection and diagnosis of the disease, to reduce its impact. The etiology

of mastitis differs between herds, and an understanding of the predominant pathogens causing infection help to direct the mastitis control program for a herd, and on an individual cow basis, support responsible antimicrobial stewardship. The purpose of this dissertation is to consider on-farm approaches to reduce the severity of mastitis or assist in its detection.

The first study, presented in Chapter 2, concentrated on prevention of mastitis, focusing vaccination against coliform mastitis, a major cause of bovine mastitis. The objective was to evaluate the efficacy of 2 commercially available J5 vaccines in dairy cows, following a mid-lactation intramammary challenge with *E. coli*. The clinical, behavioral and antibody and cytokine responses were assessed. We hypothesized that the vaccinated cows would have reduced severity and duration of CM and greater antibody levels, compared with unvaccinated controls.

The next series of studies focused on mastitis detection and diagnosis, with the overall goal of using milk and activity data to detect CM and indicate the type of pathogen responsible for infection. Considering the physiological effects of mastitis on the mammary gland and milk composition, and the flow on effects on cow demeanor or behavior, inclusion of a larger variety of sensor inputs that represent milk composition and cow behavior were expected to improve CM detection.

In Chapter 3, the objective was to evaluate time series changes in individual milk component or activity variables for all CM cases, and for Gram-negative and Gram-positive CM, and cases with no pathogen isolated. A combination of milk and activity parameters were then used to derive multiple regression models that could identify CM caused by each of the pathogen types of interest, where 3 different days, relative to CM detection, were used as baselines for time series comparisons. We expected that modeling pathogen types separately would generate more successful CM detection models than all CM cases grouped together.

In Chapter 4, the objective was to evaluate the multiple regression models derived in Chapter 3, against an independent test dataset, to investigate repeatability of the models and confirm the most appropriate baselines that captured the time series changes for the different pathogens types. The hypothesis was that indication of Gram-positive CM would be best when data as much as 10 d before CM was used, whereas the baseline for Gram-negative CM and cases with no pathogen isolated would be closer to CM detection (i.e., 3 d prior to CM) for greater success in detecting CM caused by these specific pathogen types.

In Chapter, 5, the study objective was to derive and test multiple regression models in real-time, across 2 farms. Models were tested for their ability to detect CM or IMI on the model training herd and a second independent herd. Similar to earlier studies, we hypothesized that detection performance would be improved when categorizing types of CM by Gram-status, because the models would account for the specific changes occurring before a Gram-negative or Gram-positive case of CM.

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CHAPTER 2. The effect of two J5 vaccines on clinical, behavioral and antibody response following intramammary challenge with *Escherichia coli* in mid-lactation dairy cows

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J5 vaccine efficacy by Steele. This study evaluates 2 J5 vaccines following induced *Escherichia coli* mastitis in mid-lactation dairy cows. Few differences were observed between vaccinated and unvaccinated cows, except that vaccinated cows exhibited elevated temperatures 3 h earlier and spent more time lying per rest bout than controls following intramammary challenge. Antibody concentrations differed between the 2 vaccinated groups, but this difference did not correspond to improved clinical outcomes. Our results suggest that the effects of vaccination were diminished by peak lactation.

ABSTRACT

Vaccination against coliform mastitis has become part of mastitis control programs in the past 3 decades as a means of reducing the severity of clinical mastitis. Our study objective was to evaluate the effect of 2 commercially available vaccines on clinical, behavioral and antibody response following *Escherichia coli* intramammary challenge in mid-lactation cows. Cows (n = 12 per group) were vaccinated with Vaccine 1 (V1) or Vaccine 2 (V2) at dry off, 21 d pre-calving and 14 d post-calving. Twelve cows served as unvaccinated controls (**CTL**). Cows were challenged with *E. coli* in a rear quarter at approximately 100 DIM. Milk samples were collected pre- and post-challenge for *E. coli* enumeration and SCC determination. Serum was collected to study antibody response following vaccination and challenge. Milk IgA and TNF-α concentration were determined in whey. Vaginal temperature, cow activity (including steps/h, lying time, number of lying bouts and lying bout duration) as well as milk yield and components were monitored daily. Bacterial count, SCS, milk yield and component decline, vaginal temperature, activity measures, and antibody and cytokine response were analyzed for treatment differences. The effects of parity, breed and a repeated measure of time were also tested. Seven cows had to be removed from the study for antibiotic treatment (CTL and V1, n = 3 each; V2, n = 1), 2 of which were euthanized (both CTL). Vaccinated cows exhibited fever (vaginal temperature ≥ 39.4 °C) 3 h earlier than CTL cows, but no difference in bacterial counts, SCS, and milk yield decline between treatments was observed. Vaccinated cows spent more time lying per rest bout 2 d post-challenge, but total daily lying time was not different to CTL cows during the 7 d post-challenge. The 2 vaccines differed in the antibody response as V1 cows had greater serum IgG1 and IgG2 following challenge. A parity effect was evident as primiparous cows had lower bacterial counts, SCS and a smaller milk yield decline than multiparous cows, but also had lower antibody production. The discrepancy between clinical response and antibody levels potentially suggests a role for cellular immunity in defense against

induced *E. coli* mastitis in mid-lactation dairy cows. Immunization with either J5 vaccine did not reduce clinical signs of mastitis in cows challenged at 100 DIM, demonstrating that the effects of J5 vaccination had diminished by peak lactation.

Keywords: vaccine, immune response, mastitis, parity

INTRODUCTION

Coliforms such as *Escherichia coli* and *Klebsiella pneumoniae* are frequent causes of bovine mastitis. The severity of infection can range from an acute mild or moderate case with short-term impacts on milk production and milk quality, and a relatively quick recovery, to a severe systemic illness, which can lead to death. In general, approximately 25% of cows in a well-managed herd develop clinical mastitis caused by coliform bacteria, and most of these cases occur in early lactation (Hogan and Smith, 2003). A single case of clinical mastitis occurring in the first 30 days of lactation has been estimated to cost $444 (Rollin et al., 2015). As such, vaccination of dairy cattle with a J5 bacterin has been an approach to reduce the severity of coliform mastitis for the past 30 years (Gonzalez et al., 1989). In the US, an estimated 18% of all dairy operations and 51% of large dairy herds (> 500 cows) have adopted a vaccination program against coliform mastitis (USDA, 2016).

The sudden and acute response associated with clinical mastitis caused by coliforms is due to lipopolysaccharide (**LPS**), a cell wall component of Gram-negative bacteria released upon bacterial death and a potent immune stimulator. The strain often incorporated in coliform vaccines is *E. coli* O111:B4 (J5), a rough mutant that lacks the O-polysaccharide chains of the LPS, exposing the core antigens. Immunization with a J5 vaccine produces cross-reactive antibodies with other coliform pathogens as the target is a highly conserved region of LPS, providing non-specific immunity against these pathogens. Previous studies have demonstrated reduced severity and duration of CM in mild (Hogan et al., 1995) and moderate experimental *E. coli* mastitis (Tomita et al., 1998; Hogan et al., 2005), or reduced incidence of naturally occurring clinical mastitis (Gonzalez et al., 1989; Hogan et al., 1992b) in cows immunized with a J5 vaccine. In contrast, some studies have shown few clinical differences following an *E. coli* intramammary challenge, but an antibody response was observed as a result of vaccination with a J5 bacterin (Smith et al., 1999; Tomita et al., 2000).

Our study objective was to examine the effect of 2 commercially available J5 vaccines on clinical, behavioral and antibody responses following *E. coli* intramammary challenge in mid-lactation dairy cows. Most challenge studies have used early lactation animals, whereas we proposed to investigate the effects of vaccination in cows administered the challenge during mid-lactation. Additionally, few studies have reported on behavioral measures of cows administered different J5 vaccines and a subsequent intramammary challenge. We hypothesized that vaccinated cows would show less severe signs of clinical mastitis in terms of milk appearance, bacterial count, SCC and febrile response, fewer changes in lying behavior, and have greater antibody production than unvaccinated controls. Secondly, it was anticipated that a similar antibody response would be elicited by the 2 vaccines.

MATERIALS AND METHODS

This experiment was conducted at the Dairy Science Complex – Kentland Farm in Blacksburg, Virginia. All experimental procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (Protocol #16-109).

Study Design and Animal Selection

Approximately 60 d prior to expected calving, cows and heifers (n = 65) were randomly assigned to 1 of 3 groups; Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**). Randomization was performed using the random function in Microsoft Excel (Microsoft Corporation, Redmond, WA). Lactating cows were dried off through abrupt cessation approximately 60 days prior to expected calving, and each quarter was administered a dry cow antibiotic (300 mg cephapirin benzathine; ToMORROW ®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO). Pregnant dry cows and heifers were housed on pasture during the non-lactating period until 3 wk prior to expected calving, when they were moved to a compost bedded pack barn. After calving, cows were milked twice daily in a double 12 parallel parlor at 12 h intervals and housed in a sand bedded freestall barn.

Vaccines were administered subcutaneously in the neck in 3 doses (5 mL each), following manufacturer's recommendations. The vaccination schedule for cows receiving the first 2 doses of V1 and V2 was 60 and 21 d prior to calving. At 61 ± 7 d (mean ± SD) and 18 ± 5 d prior to calving, vaccines were administered to cows in the V1 and V2 groups. The third dose of V1 was to be administered < 14 DIM and the third dose of V2 was to be given > 14 DIM. For V1 cows, the third vaccine was administered at 12 ± 1 d postpartum (range 10 to 14 d), and for V2 cows, the third vaccine was administered at 15 ± 2 d postpartum (range 13 to 18 d) and.

From the 65 enrolled cows, 36 cows were selected for an intramammary challenge with *E. coli* (mean: 102 ± 15 days in milk; range 72 to 137 d). Of the 36 cows, 23 were multiparous and 13 primiparous, and 23 were Holstein and 13 were Jersey. Selected cows were required to have no history of clinical mastitis in the current lactation prior to challenge. Two enrolled cows retained fetal membranes after calving, but the effect on the study was considered negligible as all cows were challenged after 70 DIM. Cows were pre-screened for intramammary infections at d -7, -5, and -3 relative to challenge. Milk was aseptically collected from each quarter for standard microbiological analyses (Middleton et al., 2017), described in more detail in a later section. Milk samples were submitted to a DHIA laboratory for SCC analysis (United DHIA, Radford, VA, USA). The quarter to be challenged was required to have a SCC < 100,000 cells/mL and be clear of any intramammary infection in at least 2 of 3 consecutive samples collected in the 1 wk preceding challenge.

Intramammary challenge

The intramammary challenge strain was *E. coli* 727, a well-characterized strain originally isolated from a naturally occurring IMI (Hogan et al., 1995). Previous studies report mild clinical mastitis following intramammary infusion with this strain (Hogan et al., 1992a; Yeiser et al., 2012). The challenge inoculum was prepared using a frozen stock culture streaked for isolation on esculin blood agar (**EBA**) and incubated at 37 °C overnight. A single colony was

transferred into 25 mL of trypticase soy broth (**TSB**) and grown at 37 °C for 12 h at 200 rpm to reach stationary phase. The broth culture was centrifuged at 3,000 rpm for 10 min at 4°C and the pellet resuspended in 20 mL of phosphate buffered saline (PBS; 1% solution made from Dulbecco's PBS 10%; Life Technologies Corporation, Grand Island, NY, USA). The broth culture was adjusted to 70 to 72% transmission at 540 nm, and 6 serial 10-fold dilutions in PBS were made to achieve approximately 100 cfu/mL in the final dilution. The concentration was determined by drop plating 25 µL in triplicate on EBA and counting colonies after overnight incubation at 37 °C. Before infusion, teats were cleaned with cotton balls soaked in 70% ethanol. The challenge dose (1 mL) was administered in a rear quarter via a teat canula (Jorgensen Laboratories Inc., Loveland, CO, USA), immediately following the afternoon milking.

Milk sample collection

As previously mentioned, foremilk was aseptically collected from all quarters at -7, -5 and -3 d relative to challenge, to determine quarter SCC and bacteriological status for cow selection. For cows receiving the intramammary challenge, milk was collected from all quarters at d 0 (immediately prior to challenge), and 1, 2, 3, 4, 5, 6, 7, 30, and 60 d post-intramammary challenge **(PIC)** and additionally from the challenged quarter at 6, 12, 15, 18, and 21 h PIC for bacterial identification and enumeration, and SCC determination. Bacterial identifications were completed for all aseptically collected milk samples following NMC guidelines (Middleton et al., 2017). Briefly, quarter milk samples were streaked on a quadrant of EBA and a half of MacConkey agar and incubated for 48 h at 37 °C. Growth was recorded at 24 h and 48 h. Biochemical tests were used to confirm bacterial species.

Bacterial enumeration and SCC

Bacterial enumeration was completed for milk from the challenged quarter at each sampling point (6, 12, 15, 18, and 21 h and 1, 2, 3, 4, 5, 6, and 7 d) in the 7 d PIC. Five serial 10-fold

dilutions were made in a 96-well plate for each milk sample in duplicate, using PBS as the diluent. For each dilution and duplicate, 4 replicates of 10 µL each were dropped on the surface of MacConkey agar and, after overnight incubation at 37 °C, the number of colonies were counted at the appropriate dilution and averaged. Pour plates were used for lower limit of detection, where 1 mL and 0.1 mL of undiluted milk were dispensed in 12 mL of liquid MacConkey agar in duplicate, and colonies were counted after overnight incubation at 37 °C. The bacterial counts were expressed as the number of colony forming units per milliliter of milk. Samples were sent to the Dairy Herd Information Association Laboratory (United DHIA, Radford, VA, USA) for SCC quantification using a Fossomatic™ FC (FOSS North America, Eden Prairie, MN, USA). Milk samples that was grossly clinically infected were diluted in PBS before being sent to the laboratory, with SCC calculated using the dilution factor.

Milk appearance, milk yield, and milk composition

Milk appearance was recorded to assess clinical status at the time of milk sample collection on a scale of 1 to 5, as previously described by Hogan et al. (1995): 1 = milk and quarter normal; 2 = normal quarter and slight alterations to milk (e.g. few flakes); 3 = abnormal quarter (hot and/or swollen) and normal to slightly altered milk (few flakes), or, normal quarter and abnormal milk (clots, clumps, changes in milk color); 4 = abnormal quarter and milk; and 5 = swollen quarter, abnormal milk, and systemic signs of infection (elevated rectal temperature, depression, dullness). A quarter was considered clinically mastitic if the milk appearance and mammary gland was scored ≥ 3. Milk yield (kg) was measured at each milking by an inline milk meter (Afimilk Ltd., Kibbutz Afikim, Israel) starting 1 wk prior to challenge and ending 60 d PIC. Milk composition including protein, lactose and fat percent were measured by an inline milk analyzer (AfiLab, Afimilk Ltd., Kibbutz Afikim, Israel), which has been previously validated (Kaniyamattam and Devries, 2014).

Body temperature and behavior

Vaginal temperature was recorded at 15 min intervals using a temperature data logger (Star ODDI, Iceland). Temperatures were averaged every 6 h in the first 12 h PIC, every 3 h for the next 12 h, and daily thereafter for the 7 d period PIC, to match the sampling regimen for the challenged quarter. Rectal temperatures were recorded at the same schedule that the challenged quarter was sampled, and daily thereafter for the 7 d experimental period for animal monitoring purposes. Cow behavior, including activity (steps/h), lying time (min/d), lying bouts (bouts/d) and average lying bout duration (min/bout), was monitored using an accelerometer (AfiAct II, Afimilk Ltd., Kibbutz Afikim, Israel) attached to a rear leg on each cow. Data were transmitted at each milking to the herd management software (AfiFarm, Afimilk Ltd., Kibbutz Afikim, Israel) for data storage. Behavioral data were summarized into daily periods, beginning at -1 d relative to challenge and ending 7 d PIC. A daily period consisted of 24 h relative to the time of day that challenge occurred, so that d -1 represented the 24 h immediately preceding challenge, and d 0 represented the 24 h immediately following challenge, and so on.

Determination of antibody and cytokine concentrations

At d -60, d -21 and d 14 relative to calving, blood was collected from the caudal vein of all enrolled animals (n = 65), prior to vaccine administration for vaccinated cows. For cows selected for intramammary challenge (n = 36), blood and milk samples were collected at d 0, 1, 2, 3, 6, 30 and 60 relative to challenge. After collection, blood was centrifuged (700 × *g*) for 15 min, and 4 aliquots of serum were stored at -20 °C for subsequent analysis. Milk samples were centrifuged (2,500 × *g*) for 30 min at 4 °C, and the fat layer was removed with a spatula. Four aliquots of skim milk were made in microcentrifuge tubes and stored at -20 °C.

Relative antibody concentrations to *E. coli* J5 whole cell antigens in serum and milk were determined by ELISA (Tyler et al., 1991). Isotypes assayed in serum were IgG1, IgG2, IgA and IgM at the following time points: d -60, d -21 and d 14 relative to calving, and d 0, 1, 2, 3, 6, 30 and 60 relative to challenge. An IgA assay was completed in milk collected at d 0, 1, 2,

3, 6, 30 and 60 relative to challenge. For preparation of the whole cell solution for coating plates, *E. coli* 727 was grown from stock culture to stationary phase, as for the intramammary challenge preparation. The culture was checked for sterility, formalin fixed, and checked for viability. The whole cell solution was diluted in PBS with 0.005% phenol, adjusted to 25% transmission at 610 nm, and stored at -20°C. Flat bottom 96-well microtiter plates (Immulon-1B, Thermo Scientific, Rochester, NY, USA) were coated with 100 µL whole cell stock solution in each well, sealed and incubated overnight at 37 °C. Remaining solution was decanted and plates were washed 3x with the plate wash solution (PBS + 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA)). Each 96 well plate had a negative (Fetal Bovine Serum; Atlanta Biologicals, Lawrenceville, GA, USA) and positive control (serum from a J5-immunized cow), and each sample in triplicate. The same cow's serum was used as the positive control in all assays. Serial dilutions of sample and controls in blocking buffer (PBS + 0.05% Tween 20) were prepared in a dummy plate, and 100 µL from each well was transferred to the coated plate. The plate was incubated for at least 30 min at 37 °C to allow antibody in the sample to bind to the coated antigen. The plate was washed 3x, with a 3 min incubation step between the second and third wash. Secondary antibodies for IgG1, IgG2 (sheep anti-bovine IG1/IgG2 secondary antibody, horseradish peroxidase (HRP) conjugated; Invitrogen, Rockford, IL, USA), IgA (sheep anti-bovine IgA HRP conjugated; Bethyl Laboratories, Inc., Montgomery, TX, USA) and IgM (rabbit anti-bovine IgM HRP conjugated; Bethyl Laboratories, Inc., Montgomery, TX, USA) were prepared to working concentrations according to manufacturer's recommendations, and 100 µL was added to each well before a 30 min incubation at 37 °C. Seven plate washes followed the incubation step, with 3 min incubation steps at room temperature occurring between the fourth, fifth and sixth wash. Next, 100 µL of substrate (1-Step™ ABTS; Thermofisher Scientific, Rockford, IL, USA) was added to each well and was left to develop at room temperature. Stopping reagent (1% Sodium

Dodecyl Sulfate, from 10% solution; Fisher Scientific, Fair Lawn, NJ, USA) was added after a set period, ranging between 6 and 25 min, depending which antibody was being assayed. The optical density (OD) was read at 410 nm and triplicates within each dilution for each sample were averaged. The most appropriate dilution was selected at the highest dilution within the linear phase (i.e., OD value between 0.3 and 0.8). Samples were repeated if the intra-assay coefficient of variation (CV) was > 15%. Similarly, plates were repeated if the inter-assay CV for the positive control on each plate exceeded 15%. Sample OD's were expressed relative to the positive control OD in arbitrary units.

Tumor necrosis factor-α was quantified (pg/mL) from milk samples collected on d 0, 1, 2, 3, and 6 relative to intramammary challenge using a bovine ELISA (DuoSet ELISA, R&D systems, Inc., Minneapolis, MN) according to manufacturer's instructions. In brief, flat-bottom, high-binding 96-well plates were coated with 100 μL of goat anti-bovine TNF-α capture antibody, diluted in PBS, and were incubated overnight at room temperature. Coated plates were aspirated and washed 3x (0.05% Tween 20 in PBS). Next, plates were blocked for 1 h with 300 μL of the reagent diluent (5% Tween 20 in PBS), then aspirated and washed 3x. Standards and milk samples were loaded onto the plate in duplicate. Using 2-fold dilutions in reagent diluent, a 7-point recombinant bovine TNF-α standard was loaded onto each plate, with the highest concentration of 8,000 pg/mL, and the lowest of 125 pg/mL. Following a 2 h room temperature incubation, plates were aspirated, washed 3x, and 100 μL of biotinylated goat anti-bovine TNF-α detection antibody, diluted in reagent diluent with 2% normal goat serum, was added to each well. Plates were again incubated for 2 h at room temperature, aspirated, and washed 3x. Next, 100 μL of streptavidin-HRP, diluted in reagent diluent, was added to each well and incubated for 20 min at room temperature in the absence of light. Following another 3x wash step, 100 μL of the substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) was added to each well and incubated in the dark for 20 min at room temperature. Finally, 50

μL of the stop solution (2 N H_2SO_4) was added to each well, and the OD was determined at 450 nm using a microplate reader. Duplicate readings were averaged, and a 4-parameter logistic regression standard curve was used to determine the quantity of TNF-α in each sample. If the OD reading from the milk sample did not fall on the standard curve, the milk sample was diluted in reagent diluent, repeated, and quantified appropriately using the dilution factor. Samples were also repeated if the intra-assay CV was > 15%.

Statistical analysis

Bacterial counts from the challenged quarter were transformed into $\log_{10}$ cfu/mL. Quarter SCC was transformed to somatic cell score (SCS), which uses a base-2 log-transformation [SCS = $\log_2$ (SCC/100,000) + 3]. Milk yield and milk components PIC were expressed as the percent change when compared to the 7 d mean of these measures from the week preceding challenge. Relative OD for all antibody and TNF-α assays were transformed to natural logarithm. For all models, residuals were analyzed for normality and outliers. Outliers were removed if the studentized residuals exceeded 4. Mortality and morbidity rates were analyzed using the MIXED procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC) with the main effects of treatment (V1, V2, CTL), parity (multiparous or primiparous), and breed (Holstein or Jersey) offered into the model and a binary outcome of 0 or 1 (0 indicating survival or successful completion of the trial without treatment, and 1 indicating death or treatment required for the mortality and morbidity models, respectively).

Vaginal temperature, log cfu/mL, SCS, milk appearance, relative milk yield and components, behavioral variables and antibody and cytokine responses were analyzed for treatment differences using the GLIMMIX procedure in SAS. Variables offered into each of the models included the main effects of treatment, parity, breed, a repeated measure (period), and associated 2-way interactions with cow modeled as a random effect. For the behavioral responses, including steps/h, number of rest bouts, min spent lying and average rest duration,

the day preceding challenge was used as a covariate to account for cow differences for those behaviors. Six cows did not have covariate data and were not included in the behavioral models. The antibody models included ln serum IgG1, IgG2, the ratio of IgG1 to IgG2, IgM, and IgA, and milk IgA. The ln relative OD of each antibody at d -60, prior to initial vaccination, was used as a covariate for each respective serum antibody model to account for differences in individual cows prior to commencing the vaccination schedule for this study. Days in milk was checked as a covariate for milk yield, milk component, behavioral and antibody models, but was not significant. Repeated measures, such as day or period, depending on the response variable, were modeled using the autoregressive error structure and the Kenward-Rogers procedure was used for degrees of freedom approximation. Backwards elimination was used to eliminate non-significant terms from highest to least significant until all variables were significant ($P \leq 0.05$); however, the main effect of treatment was forced into the model, regardless of significance. Main effects were retained in the model if $P \leq 0.05$ or if the main effect was involved in a significant interaction term. Least squares means and standard errors for each model were estimated for significant variables, and Tukey adjusted *P*-values were declared significant at $P \leq 0.05$.

RESULTS

Thirty-six animals received the intramammary challenge of 113 ± 13 cfu/mL of *E. coli* in 1 rear quarter. Challenged animals in each treatment group are summarized for breed and parity in Table 2.1. Seven cows were removed from the study for antibiotic treatment during the 7 d post-challenge period; 3 each from V1 and CTL groups, and 1 V2 cow. Of these, 2 CTL cows were euthanized. The need for veterinary attention was determined without regard to vaccination status. Data collection from cows ceased at the time they were treated and removed from the study, but data collected prior to removal remained in analyses. Treatment had no

effect on mortality rate ($P = 0.18$) or morbidity rate in the 7 d PIC ($P = 0.33$), but the morbidity rate was greater in multiparous cows compared with primiparous heifers ($P = 0.02$).

Febrile response

Mean vaginal temperatures were elevated (≥ 39.4 °C) at 12 h PIC for both V1 (39.7 °C ± 0.1) and V2 (39.7 °C ± 0.1) cows, whereas CTL cows had lower temperatures at this time (38.9 °C ± 0.1; $P < 0.01$; Figure 2.1). Temperatures peaked at 15 h PIC, returning to within the normal range by 21 h PIC. No differences in body temperature were observed between groups from 15 h PIC, and for the remainder of the 7 d post-challenge period.

Milk-associated parameters

All but 1 cow developed clinical mastitis (score ≥ 3) in the challenged quarter by 12 h PIC, with the remaining cow having visible clinical mastitis by 15 h PIC. Clinical signs of mastitis remained apparent in challenged quarters until 5 d PIC, where the average milk appearance score dropped below 3. A parity effect on milk appearance was observed as multiparous cows took 2 d longer to return to normal milk appearance (score 1 or 2), with greater scores than primiparous heifers from 3 to 7 d PIC ($P < 0.01$; Table 2.2).

Bacterial counts and SCS did not differ between treatment groups for the study period, but a parity by period interaction was observed for both responses (Table 2.2). Multiparous and primiparous animals both peaked in *E. coli* concentration at 12 h PIC, with multiparous cows having significantly greater bacterial counts than primiparous heifers from 15 h to 5 d PIC ($P < 0.05$; Figure 2.2A). Somatic cell score peaked between h 18 and d 2 PIC, and began to decline by 3 d PIC, though never returning to pre-challenge levels in the 60 d following challenge, especially for multiparous animals. Primiparous heifers had greater peak SCS at 24 h PIC, but their SCS declined faster, with significantly lower SCS observed at d 3, 6, 7, 30 and 60 PIC, compared with multiparous cows ($P < 0.05$; Figure 2.2B). A breed by period interaction was

also noted for SCS, where initially, Jersey cows had lower SCS at 15 h PIC, but later, SCS was significantly greater than Holsteins at d 1 and d 60 PIC ($P = 0.01$; data not shown).

No treatment differences were observed in the milk yield response following intramammary challenge with *E. coli*. In the first 24 h PIC, mean milk yield across all challenged animals declined to 62% of the 7 d pre-challenge baseline, with the largest decline occurring at 2 d PIC, where cows produced 49% of pre-challenge milk yield. A steady recovery in milk yield was observed, reaching 86% of pre-challenge levels by 7 d PIC, and 93 to 94% by 30 and 60 d PIC (Figure 2.3). Parity was significantly associated with the milk yield response to challenge, as multiparous cows had a greater decline in milk yield compared with primiparous heifers($P < 0.01$; Table 2.2).

Milk components including fat, protein and lactose yields were not different by treatment group, but declined for 2 d following intramammary challenge, and steadily recovered thereafter (Figure 2.3). Similar to milk yield, parity had an effect on the 3 milk components tested, as multiparous cows had a greater decline in yields (relative to pre-challenge baseline milk fat, protein and lactose yields) than primiparous heifers (Table 2.2). Significant treatment by parity and treatment by breed interactions were observed for milk protein production. Multiparous cows treated with V1 had a greater reduction in milk protein yield (55.9 ± 5.8% of pre-challenge protein yield) compared with multiparous CTL cows (77.8 ± 4.5%; $P = 0.02$). Holstein animals treated with V1 declined more in milk protein production (64.7 ± 5.4% of the pre-challenge baseline yield) over the 7 d PIC compared with V2 Holstein animals (80.4 ± 3.6%; $P = 0.05$).

Behavioral parameters

For the behavioral measures, the respective 1 d pre-challenge covariates were significant in all models, including step activity, lying time, lying bout duration, and no. of lying bouts.

Treatment and parity least squares means and interactions with period are presented in Table 2.2. Animals vaccinated with V1 or V2 spent more time lying per bout than CTL animals on d 2 PIC (P = 0.01; Figure 2.4). A treatment by parity interaction was observed for lying bout duration, where multiparous V1 cows spent more time lying per bout than multiparous CTL cows (70.7 ± 4.9 vs. 56.1 ± 3.5 min/bout; P = 0.05). Step activity was not different between treatments, except when comparing Jersey animals. Jersey cows treated with V2 were less active than V1 Jersey cows (P = 0.01; Figure 2.5A). Primiparous heifers were more active than multiparous cows (P = 0.04; Table 2.2). A treatment by parity interaction was observed for the number of lying bouts per day; primiparous heifers treated with V1 had more lying bouts than V2 and CTL heifers (P < 0.01; Figure 2.5B). The opposite trend was seen for multiparous cows; V1 cows had fewer lying bouts than V2 and CTL cows ($P \leq 0.01$; Figure 2.5B). Lying time was not associated with treatment or parity (Table 2.2).

Inflammatory parameters

The respective serum antibody level measured prior to the first vaccination (d -60 relative to calving) was a significant covariate in the IgG1, IgG2, IgA and IgM models. The serum IgG1 concentration (i.e., relative OD) in V1 cows was greater than in CTL cows (P < 0.05; Figure 2.6A). Multiparous cows had greater serum IgG1 levels compared with primiparous heifers at d -21 relative to calving and at d 6, 30 and 60 PIC (Figure 2.7A).

Serum IgG2 concentration differed between treatment groups (P < 0.01) and between treatments over time (P < 0.01; Table 2.2). Animals vaccinated with V1 had more serum IgG2 than V2 and CTL animals at 14 DIM (prior to the third vaccination; P < 0.01) and at d 0, immediately prior to challenge ($P \leq 0.06$; Figure 2.6B). Greater serum IgG2 levels were maintained in V1 cows for all time points within the 60 d PIC, compared with V2 cows (P < 0.05). Serum IgG2 concentrations in V1 cows were also greater than in CTL animals at d 1, 30 and 60 PIC (P < 0.05). At d 6 PIC, serum IgG2 concentrations in CTL cows exceeded that of

V2 treated cows ($P < 0.01$). Multiparous cows had greater serum IgG2 levels than primiparous heifers at d 6, 30 and 60 PIC ($P < 0.01$; Figure 2.7B). Across all time points, serum IgG2 levels in Holsteins were similar, regardless of parity, whereas multiparous Jersey cows had greater serum IgG2 levels than primiparous Jersey heifers ($P < 0.01$). Multiparous Jersey cows also had a greater serum IgG2 concentration than their Holstein multiparous counterparts ($P < 0.05$). The ratio of serum IgG1 to IgG2 in V2 animals exceeded that of V1 animals at 14 DIM, and was greater than the IgG1:IgG2 ratio in both V1 and CTL animals at 6 d PIC ($P < 0.01$; Figure 2.6C). At 21 d pre-partum, multiparous cows had greater serum IgG1:IgG2 than primiparous heifers ($P < 0.01$), but the opposite was seen at 14 DIM ($P = 0.01$; data not shown).

No effects of treatment on serum IgM or IgA levels were observed (Table 2.2). Multiparous cows had more serum IgM and serum IgA than primiparous heifers at d 6, 30 and 60 PIC ($P < 0.01$; Figure 2.7C and 2.7D). Jersey animals had greater serum IgM concentrations than Holstein animals at 14 DIM (prior to the third vaccination), and at d 1, 2, 30 and 60 PIC ($P < 0.05$; data not shown). Jersey animals had more serum IgA than their Holstein counterparts at 14 DIM, and at 30 and 60 d PIC ($P < 0.01$), but at 6 d PIC, the reverse was observed with serum IgA levels in Holsteins exceeding that of Jersey animals ($P < 0.05$; data not shown).

The milk IgA concentrations were similar between treatments from d 0 to d 6 relative to challenge, but V1 animals had greater milk IgA levels at d 30 and 60 PIC, compared with V2 and CTL animals ($P < 0.05$; Figure 2.6D). Multiparous cows had greater milk IgA levels than primiparous heifers before challenge (d 0), and at d 3, 6, 30 and 60 PIC ($P < 0.05$; Figure 2.8). Jersey animals had greater milk IgA levels than Holstein animals at d 0, and at d 60 PIC ($P < 0.05$; data not shown). The levels of TNF-α in milk increased by almost 30-fold from d 0 to d 1, and steadily declined over time to 7-fold greater than pre-challenge levels by 6 d PIC ($P < 0.01$; data not shown). No differences in milk TNF-α levels were observed between treatment

groups, but milk TNF-α levels were greater in multiparous cows, compared with primiparous heifers (Table 2.2).

DISCUSSION

We hypothesized that vaccinated animals would have reduced severity and duration of clinical mastitis compared with unvaccinated controls following intramammary challenge with *E. coli* in mid-lactation dairy cows. Further, we expected that the 2 vaccines (V1 and V2) would provide similar protection to *E. coli* challenge and, as such, cows receiving either vaccine would have comparable clinical, behavioral and antibody responses following challenge. All cows in our study developed clinical mastitis in the challenged quarter and showed signs of systemic illness 15 h PIC; therefore, a moderate to severe infection was established. The concentration of *E. coli* recovered from milk of challenged quarters peaked at 12 h PIC, SCS peaked shortly thereafter at 18 h PIC, and milk production was halved at 2 d PIC, following a similar response to a previously described *E. coli* intramammary challenge (Tomita et al., 2000).

Contrary to our hypothesis, little difference was observed between vaccinates and controls in clinical response to *E. coli*. Vaccinated animals developed fever 3 hours earlier than CTL animals, but no differences between treatments were observed in quarter bacterial count, milk appearance or SCS, or in milk yield and components measured at the cow level, other than protein yield. Earlier fever would typically indicate a quicker induction of the inflammatory response, and systemic involvement that may be associated with faster pathogen recognition or bacterial growth (Mehrzad et al., 2008). However, whether a rapid inflammatory response corresponds to quicker recovery of clinical signs of infection depends on many cow factors (reviewed by Burvenich et al., 2003). One study in primiparous heifers demonstrated earlier peak rectal temperatures in animals given a greater intramammary challenge dose (10^6 cfu/mL vs 10^4 cfu/mL), and those given the greater dose also had a quicker recovery; however, results

may not be comparable as a different strain was used (*E. coli* P4 (O32:H37); Vangroenweghe et al., 2004). In our study, the 3 h advanced febrile response for vaccinated cows, compared with unvaccinated controls, did not correspond to a quicker recovery.

Conflicting results regarding temperature and clinical outcomes of challenge have been reported in experimental challenge studies that used the same strain as the current study (*E. coli* 727). An increase in peak temperature at 12 h PIC in control cows was associated with greater bacterial counts and SCC, and a longer duration of abnormal milk appearance (Hogan et al., 2005), whereas control cows with elevated temperature at 3 d PIC had lower bacterial counts than vaccinates, and no difference was observed in SCC or duration of clinical mastitis (Smith et al., 1999). To add further complexity, studies that demonstrated no difference in temperature between vaccinated and control cows, reported either a faster reduction in *E. coli* concentration in milk from vaccinates, and quicker milk yield recovery, but no influence of vaccination on milk appearance (Tomita et al., 1998), or greater peak bacterial counts with a faster reduction, for vaccinated cows, and shorter duration of IMI (80 h), compared with unvaccinated controls (130 h; Hogan et al., 1995). Contradictory results are not unusual in *E. coli* intramammary challenge studies, with potential explanations including: differences in vaccines and adjuvants used (Hogan et al., 2005), stage of lactation and time since last vaccination (Wilson et al., 2009), and differences in concentration and virulence of the bacterial strain being infused at challenge (Smith et al., 1999; Vangroenweghe et al., 2004). Many studies have challenged cows in the first 30 DIM, whereas cows in this study were challenged at approximately 100 DIM, possibly contributing to some of the inconsistencies observed in the clinical response to intramammary challenge compared with previous literature.

Regarding the behavioral response to *E. coli* intramammary challenge, vaccinated animals displayed less restlessness by spending more time lying per bout than CTL animals at 2 d PIC, but no treatment differences were apparent in total lying time. A large study established that

healthy cows housed in free-stalls spent 11 ± 2.1 h lying/d (660 min/d), broken up into 9 ± 3 bouts/d of 88 ± 30 min/bout in duration (Ito et al., 2009). Whilst variation exists between farms and between individual cows (Ito et al., 2009), cows in our study spent less time lying overall (mean 554 min/d), and per lying bout 1 d PIC. A recent study demonstrated more deviations in behavior in the 'pre-clinical' phase (0 to 8 h), and the 'acute' phase (12 to 24 h) following challenge with *E. coli* P4 (O32:H37), compared with the 'remission' phase, 32 to 80 h PIC (des Roches et al., 2017). Cows changed less often between lying and standing and were found to stand still for longer periods (des Roches et al., 2017). Initially, we summarized behavioral data in 3 h periods for the first 24 h to assess the immediate response following challenge. Because no differences were observed, data were summarized into 24 h periods, following the time of challenge. The reduced restlessness at 2 d PIC for vaccinated cows may indicate less discomfort compared with CTL cows. Although natural sickness behavior is thought to involve more time spent lying to conserve energy and maintain the febrile response (Johnson, 2002), studies involving mastitic cows have demonstrated reduced lying time, particularly in the early stages of an acute infection such as that caused by infusion of live *E. coli* (Yeiser et al., 2012) or LPS (Siivonen et al., 2011). The local effects of mastitis including udder swelling and increased intramammary pressure might discourage cows from lying, or lead to more frequent position changes in CTL cows. Nonetheless, no difference was seen in most of the clinical outcomes evaluated, so the reduced restlessness in vaccinated cows was not associated with quicker recovery.

Interestingly, across the 7 d study period, we observed more lying bouts per day for primiparous heifers treated with V1 (16 bouts/d) than V2 and CTL primiparous heifers, but the opposite was seen for multiparous cows, as V1 cows had fewer lying bouts (8 bouts/d) than V2 and CTL cows. The reasons behind this difference in only V1 treated animals of different parities are unknown, but a similar *E. coli* challenge study demonstrated more lying bouts/period in

primiparous heifers than multiparous cows (Yeiser et al., 2012). Step activity was only different between treatments in Jersey animals, where V2 animals were less active than V1, and tended to be less active that CTL Jersey animals. This finding was based on just 2 V2 Jersey animals, so the result is not considered to be of great biological significance.

Although a similar clinical response to intramammary challenge was observed between the 2 vaccinated groups, there was a clear disparity between vaccinated cows in antibody response. Cows treated with V1 had more serum IgG2 than V2 cows at the third vaccination (14 d post-calving) and immediately prior to challenge, through to 60 d PIC. Additionally, V1 cows had greater serum IgG1 at 1, 30 and 60 d PIC. Because cows were challenged in mid-lactation, we did not expect this difference between vaccinated groups. Antibody levels are known to decline over time, hence the need for successive vaccinations with the start of each lactation (Wilson et al., 2009). The difference in antibody levels observed between vaccines in our study may have been related to variations in the composition of the vaccine, such as the adjuvant. The role of the adjuvant is to produce a superior immune response to immunization than the antigen would produce on its own (Awate et al., 2013), but the adjuvant incorporated into each vaccine was not disclosed. Regardless, the increased antibody response of V1 cows in our study was not associated with reduced severity or quicker recovery of induced clinical mastitis.

Since the introduction of J5 vaccination in the late 80's, the mechanism of action has not been clearly established (Dosogne et al., 2002; Schukken et al., 2011). The classical hypothesis was that increased antibody titers to *E. coli* J5 in vaccinated cows enhanced opsonization of LPS, thereby neutralizing antigen, and flagging it for clearance by phagocytes (Tyler et al., 1991; Hogan et al., 1992b). Class switching of IgM to IgG1 or IgG2 has been reported in J5 vaccinated cows, where serum IgM levels were greater in unvaccinated controls (Wilson et al., 2009), but no difference in serum IgM was found between treatments in our study. In experimental mastitis studies, vaccination with a J5 bacterin often led to increased IgG titers

above unvaccinated controls, but an improvement in clinical outcomes such as reduced bacterial count and duration of clinical signs has not been consistently reported (Smith et al., 1999; Tomita et al., 2000). A growing body of evidence points to neutrophil recruitment and function having a critical role in defense against *E. coli*, in synergy with adaptive immunity (Hill, 1981; van Werven et al., 1997; Herry et al., 2017).

We observed a lower ratio of IgG1 to IgG2 for the V1 treated group, compared with V2 animals, at 14 DIM and 6 d PIC. An immune response with greater IgG2 production follows a T-helper type 1 (**Th-1**) response (Stevens et al., 1988). The Th-1 cells promote inflammation through cytokine production, which initiate class switching from IgM to IgG2. An important function of IgG2 is as an opsonin that facilitates phagocytosis by neutrophils (Stevens et al., 1988; Estes and Brown, 2002). An influx of neutrophils to the mammary gland occurs on recognition of LPS, and the speed of neutrophil mobilization was found to be important in host defense against *E. coli* (Hill et al., 1983; Burvenich et al., 2003). Neutrophil trafficking and activation is mediated by T cells, emphasizing the link between innate and cell-mediated immunity (Shafer-Weaver et al., 1999). The immune response in early lactation favors a T-helper type 2 (**Th-2**) response, with greater production of IgG1, whereas later in lactation the response shifts to Th-1 which is more protective against mastitis (Shafer-Weaver et al., 1999). Vaccination with J5 is hypothesized to reverse that trend to a pro-inflammatory state in early lactation (Schukken et al., 2011). With this in mind, the lack of clinical differences between vaccinated groups and the unvaccinated CTL animals may not be surprising, considering that cows were challenged at a time when they are biologically more resistant to mastitis, and that any advantage offered by the J5 vaccine would be diminished because the last vaccination was approximately 12 wk prior.

To further support the relative importance of cell-mediated immunity over humoral immunity, we report a parity effect, where reduced antibody levels were associated with faster clinical

recovery in primiparous heifers. Regardless of vaccination status, primiparous animals had reduced bacterial counts, lower SCS from 2 d PIC, returned to normal milk appearance 2 d earlier, and had a smaller decline in milk production and components than multiparous cows, combined with a weaker antibody response. When administered the same concentration of *E. coli* via intramammary challenge, primiparous heifers were able to clear the infection faster than multiparous cows. A greater SCS was observed in primiparous animals 24 h PIC, which declined more rapidly than for multiparous cows by 3 d PIC. Comparably, another *E. coli* 727 challenge study demonstrated faster bacterial growth in multiparous cows, and greater losses in milk production, compared with primiparous heifers (Yeiser et al., 2012). Other studies have also noted differences in severity of induced *E. coli* clinical mastitis between primiparous and multiparous animals (van Werven et al., 1997; Mehrzad et al., 2002). Reduced serum IgG1 levels were observed in primiparous heifers at the second vaccination (-21 d relative to calving), and all serum antibodies measured were reduced in primiparous animals at d 6, 30 and 60 PIC, compared with multiparous cows. It is reasonable to speculate that antibody production to J5 was lesser in primiparous cows, because younger animals have reduced exposure to coliform mastitis pathogens than multiparous cows, due to a shorter period of lactation (Wilson et al., 1997). Moreover, a lesser antibody response in primiparous heifers, combined with quicker recovery of clinical mastitis could point to a greater relative importance of cell-mediated immunity. A stronger immune response to *E. coli* in the mammary gland of primiparous animals has been partly attributed to enhanced neutrophil function and ability to clear the pathogen (Vangroenweghe et al., 2004). The bactericidal capacity of migrated neutrophils differed between primiparous and multiparous animals, with greater viability and oxidative burst activity reported in neutrophils from primiparous animals (Mehrzad et al., 2002). Still, this must be interpreted with caution, as studies of immune function typically occur

in early lactation coinciding with the period of immunosuppression commonly reported in transition dairy cows (Shafer-Weaver et al., 1999).

We observed greater concentrations of TNF-α in milk of multiparous compared with primiparous animals. In mice, neutrophil recruitment was achieved by TNF-α signalling from macrophages following recognition of *E. coli* through LPS and the pathogen recognition receptor, toll-like receptor 4 (TLR4; Elazar et al., 2010). We measured TNF-α at d 0, 1, 2, 3 and 6 PIC, but more frequent sampling in the first 24 h following challenge may have revealed a quicker increase in TNF-α in primiparous animals to coincide with the greater SCS observed 24 h PIC. Nonetheless, in multiparous cows, the greater TNF-α at the time points we measured are likely associated with the increased *E. coli* concentration at 15 h PIC and increased SCS at 3 d PIC. Gunther et al. (2010) demonstrated an association between the concentration of *E. coli* in bovine milk and the level of cytokines such as TNF-α and IL-8 induced as part of the immune response. Thus, the combination of a delay in neutrophil recruitment and potentially reduced neutrophil function are proposed as potential explanations for the increased duration of clinical signs, greater bacterial counts and longer SCS elevation seen in multiparous cows, even though antibody levels were greater.

CONCLUSIONS

Overall, the clinical responses to *E. coli* intramammary challenge in mid-lactation were not vastly different between vaccinated animals and unvaccinated controls. Vaccinated animals displayed fever 3 h earlier and less restlessness 2 d PIC, compared with control animals, but no differences were observed between treatments for quarter bacterial counts, milk appearance, and SCS, and milk yield and components at the cow level. Although animals vaccinated with V1 had greater IgG1 and IgG2 production than V2 and CTL animals, this was not accompanied by any improvement in clinical recovery from the *E. coli* challenge. Additionally, primiparous heifers produced consistently less antibodies than multiparous cows, but displayed less severe

signs of clinical mastitis during the post-challenge period. Immunization with either J5 vaccine did not reduce clinical signs of mastitis in cows challenged at 100 DIM, demonstrating that the effects of J5 vaccination had diminished by peak lactation. Additional doses may be necessary to provide longer-lasting protection against coliform mastitis later in lactation.

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Table 2.1 Number of cows in each treatment group for parity, breed, morbidity and mortality in the 7 d period following an intramammary challenge with *Escherichia coli* for cows vaccinated with Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**).

Treatment group	**Parity (no.)**		**Breed (no.)**		**Morbidity (no.)**	**Mortality (no.)**	**No. cows completed trial**
	1	**2+**	**H**	**J**			
V1	6	6	6	6	3	0	9
V2	4	8	10	2	1	0	11
CTL	8	9	7	5	1	2	9
Total	13	23	23	13	5	2	29

Table 2.2 Least squares means (± SEM) for treatment (trt: Vaccine 1 (**V1**), Vaccine (**V2**) or unvaccinated controls (**CTL**)) and parity (primiparous vs. multiparous) effects for clinical, behavioral, and inflammatory parameters following an *Escherichia coli* intramammary challenge. Milk, fat, protein, and lactose yields are expressed as percentages of the pre-challenge (**PC**) baseline yields. Serum and milk Ig parameters are expressed as natural logarithmic of the relative optical density (**ln ROD**) with a mean shift of 5, and milk tumor necrosis factor-α (**TNF-α**) is expressed as natural logarithmic of the pg/mL with a mean shift of 1. The treatment by period (h or d PC) and parity by period interactions are only presented if significant ($P < 0.05$) in the final model.

	Treatment			*P* value			Parity		*P* value		
	V1	**V2**	**CTL**	**Trt**	**Period**	**Trt × Period**	**Primiparous**	**Multiparous**	**Parity**	**Period**	**Parity × Period**
Clinical and milk-associated parameters											
Bacterial count (log cfu/mL)	2.4 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	0.82	< 0.01		1.8 ± 0.2	2.9 ± 0.2	< 0.01	< 0.01	0.01
SCS[1]	7.5 ± 0.2	7.6 ± 0.2	7.0 ± 0.2	0.16	< 0.01		7.0 ± 0.2	7.7 ± 0.2	0.01	< 0.01	< 0.01
Temperature (°C)	38.9 ± 0.0	38.9 ± 0.0	38.9 ± 0.1	0.54	< 0.01	< 0.01				< 0.01	
Milk appearance (1-5 scale)	3.0 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	0.75	< 0.01		2.8 ± 0.1	3.1 ± 0.1	< 0.01	< 0.01	< 0.01
Milk yield (% of PC yield)	75.1 ± 3.3	76.4 ± 3.1	80.2 ± 3.4	0.52	< 0.01		83.5 ± 2.9	70.9 ± 2.5	< 0.01	< 0.01	
Fat (% of PC yield)	81.3 ± 3.2	81.4 ± 3.1	86.5 ± 3.4	0.45	< 0.01		88.9 ± 2.9	77.2 ± 2.5	< 0.01	< 0.01	
Protein (% of PC yield)	73.5 ± 3.4	71.5 ± 4.6	80.8 ± 3.8	0.23	< 0.01		83.0 ± 3.6	67.6 ± 2.9	< 0.01	< 0.01	
Lactose (% of PC yield)	70.0 ± 4.0	71.9 ± 3.4	75.7 ± 3.8	0.54	< 0.01		79.1 ± 3.2	66.0 ± 3.0	< 0.01	< 0.01	
Behavioral parameters											
Activity (steps/h)	96.3 ± 3.6	81.0 ± 4.7	92.0 ± 4.1	0.04	< 0.01		95.4 ± 4.1	84.2 ± 2.9	0.04	< 0.01	
Lying time (min/d)	683 ± 21	713 ± 19	670 ± 22	0.29	< 0.01					< 0.01	
LB[2] duration (min/bout)	60.6 ± 3.0	63.3 ± 2.5	59.2 ± 3.0	0.56	< 0.01	0.04	58.6 ± 2.3	63.5 ± 2.2	0.13	< 0.01	0.01
No. LB[2] (no./d)	12.2 ± 0.7	12.2 ± 0.6	12.0 ± 0.7	0.95	< 0.01		13.0 ± 0.6	11.2 ± 0.5	0.02	< 0.01	< 0.01
Inflammatory parameters											
Serum IgG1 (ln ROD)	4.2 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	0.02	< 0.01	0.09	3.8 ± 0.1	4.1 ± 0.1	0.02	< 0.01	< 0.01
Serum IgG2 (ln ROD)	5.5 ± 0.1	4.9 ± 0.1	5.1 ± 0.1	< 0.01	< 0.01	< 0.01	5.0 ± 0.1	5.3 ± 0.1	0.03	< 0.01	< 0.01
Serum IgG1:IgG2 (ln ROD)	3.6 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	0.02	< 0.01	0.02	3.7 ± 0.1	3.8 ± 0.1	0.54	< 0.01	< 0.01
Serum IgM (ln ROD)	3.8 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	0.40	< 0.01		3.6 ± 0.1	4.0 ± 0.1	< 0.01	< 0.01	< 0.01
Serum IgA (ln ROD)	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	0.45	< 0.01		3.6 ± 0.1	4.0 ± 0.1	< 0.01	< 0.01	< 0.01
Milk IgA (ln ROD)	2.4 ± 0.2	2.0 ± 0.2	1.9 ± 0.2	0.25	< 0.01	0.04	1.6 ± 0.2	2.6 ± 0.1	< 0.01	< 0.01	< 0.01
Milk TNF-α (ln pg/mL)	5.5 ± 0.5	5.6 ± 0.5	5.2 ± 0.6	0.89	< 0.01		4.1 ± 0.5	6.8 ± 0.4	< 0.01	< 0.01	

Figure 2.1. Least squares means (± SEM) of vaginal temperature in the hours following an intramammary challenge with *Escherichia coli* for cows vaccinated with Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**). Different superscripts denote significance ($P < 0.05$) between treatment within hour.

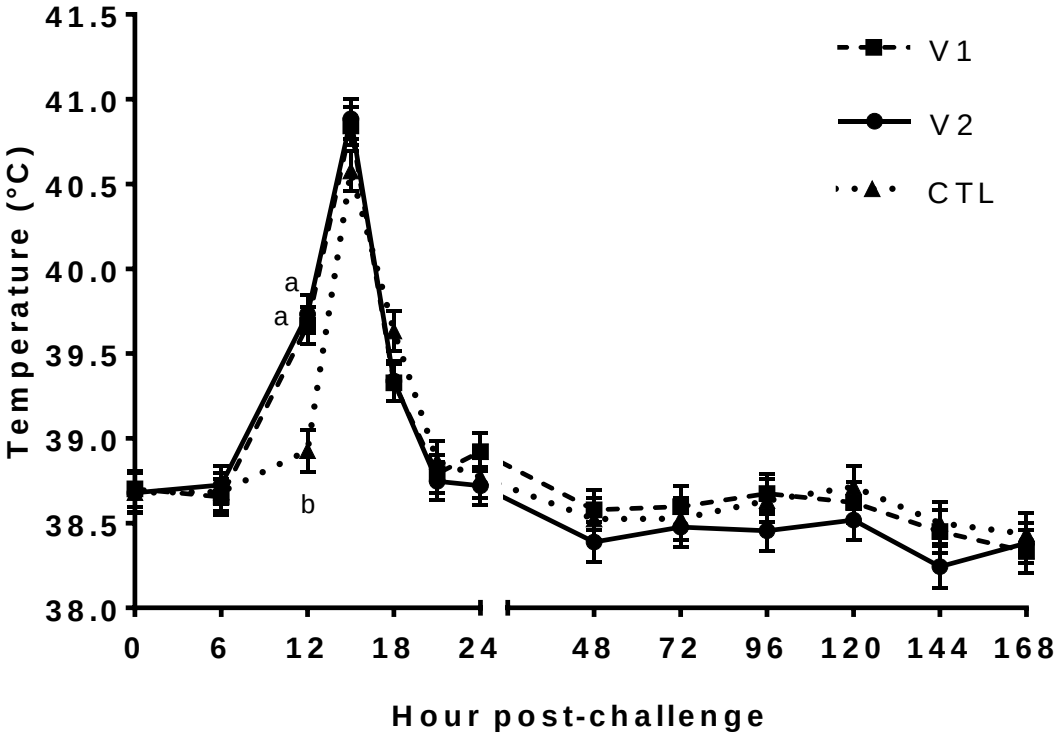


Figure 2.2. Least squares means (± SEM) of multiparous and primiparous cows for **A)** bacterial count (log cfu/ml) and **B)** somatic cell score (**SCS**) in the hours and days following intramammary challenge with *Escherichia coli*. * indicates $P < 0.05$ between parities within time**.**

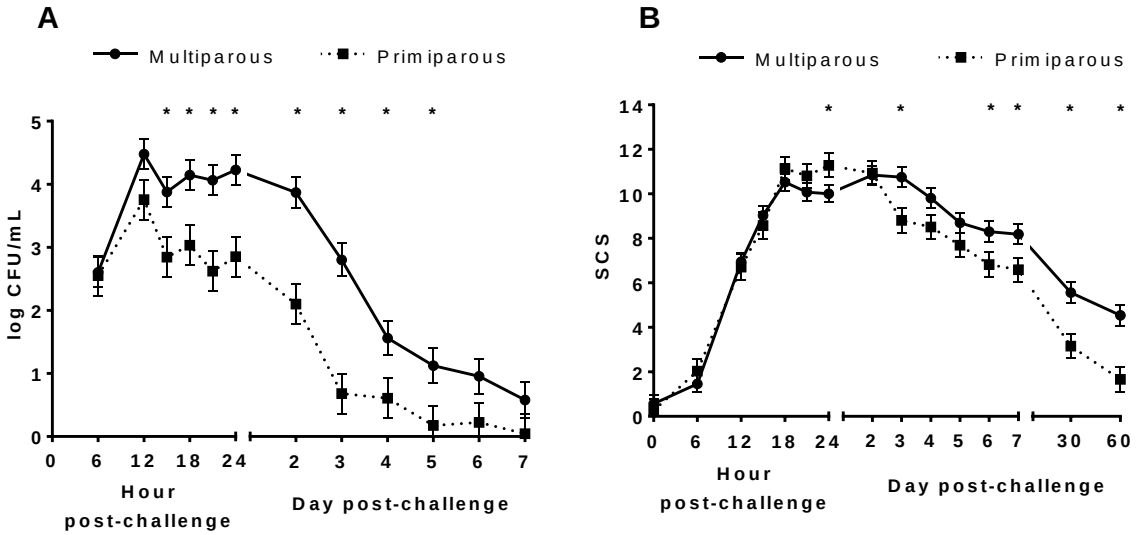


Figure 2.3. Least squares means (± SEM) of milk yield (kg), milk fat (kg), milk protein (kg) and milk lactose (kg) in the 60 d following intramammary challenge with *Escherichia coli*. Values are expressed as a percentage of the 7 d average baseline preceding challenge.

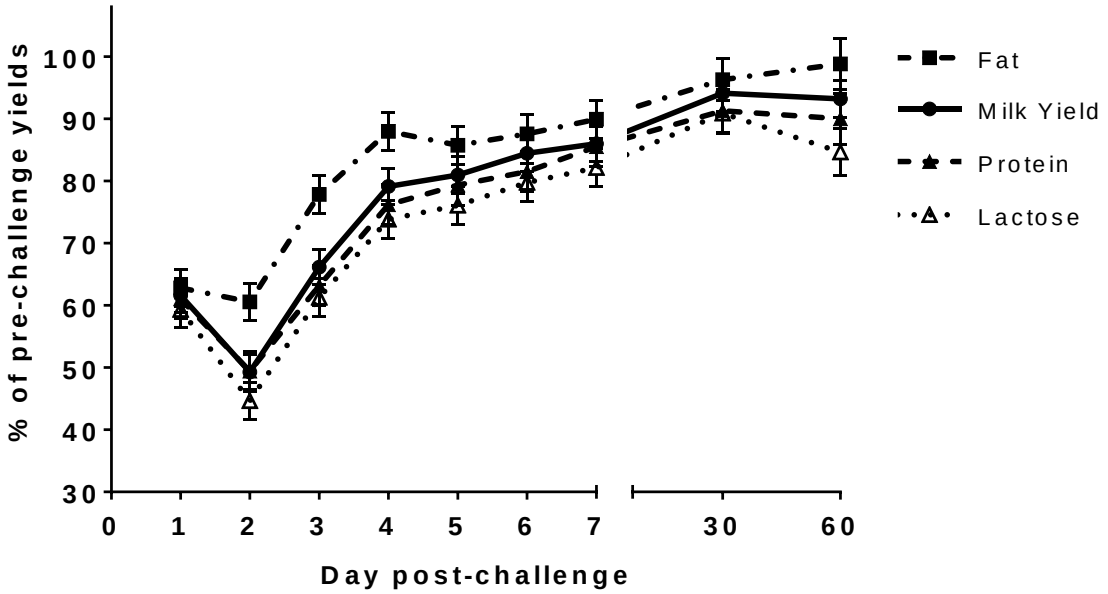


Figure 2.4. Least squares means (± SEM) of lying bout duration (min/bout) in the 7 d following an intramammary challenge with *Escherichia coli* for cows vaccinated with Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**). Different superscripts denote significance ($P < 0.05$) between treatments within day.

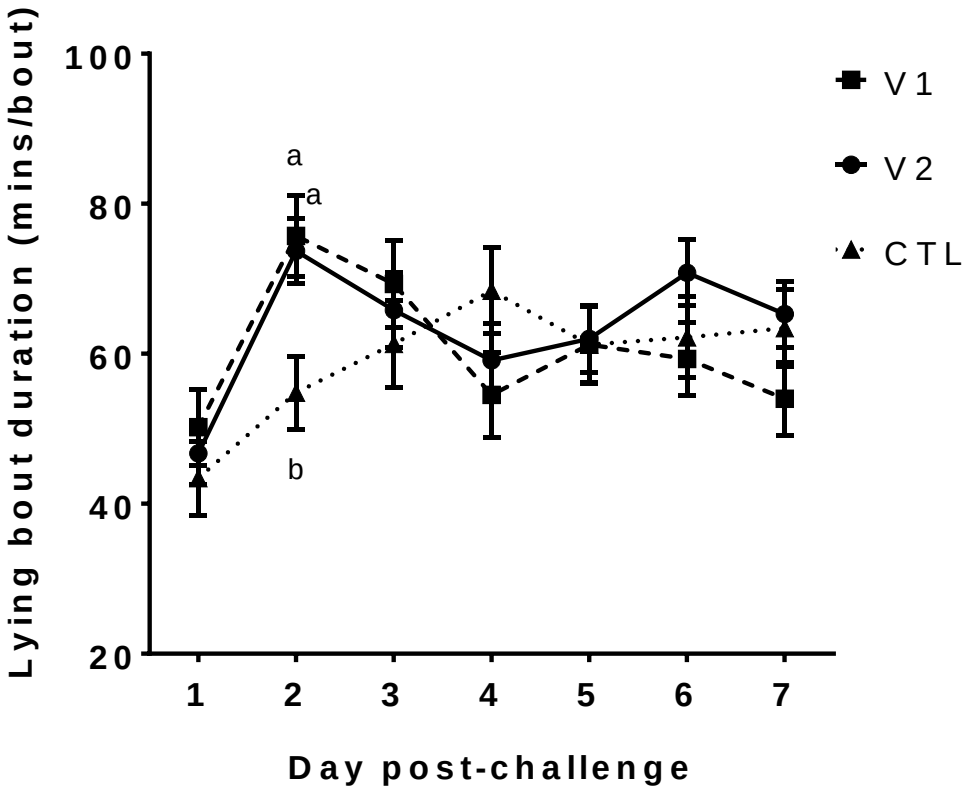


Figure 2.5. Least squares means (± SEM) of A) step activity (steps/h) for Holstein and Jersey cows and B) lying bouts (no./d) for primiparous and multiparous cows vaccinated with Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**) in the 7 d following an intramammary challenge with *Escherichia coli*. * indicates significance ($P < 0.05$) between treatment within breed or treatment within parity. Different superscripts denote significance ($P < 0.05$) between treatment within breed or parity.

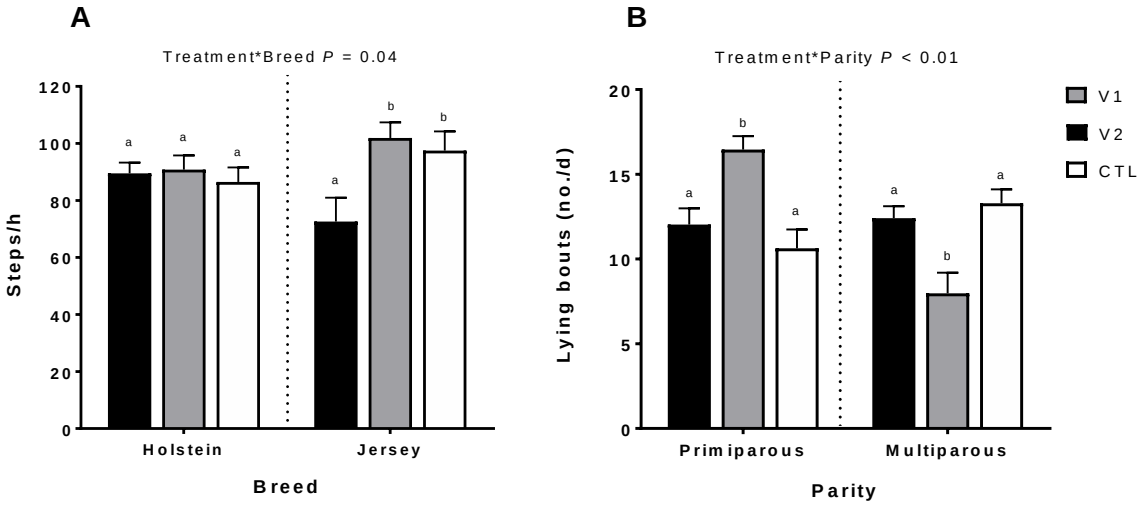


Figure 2.6. Least squares means of the ln-transformed relative optical density of A) serum IgG1, B) serum IgG2, C) serum IgG1:IgG2 ratio, and D) milk IgA for cows vaccinated with Vaccine 1 (**V1**), Vaccine 2 (**V2**), or unvaccinated controls (**CTL**) following the second and third vaccination given at d -21 and d 14 relative to calving, respectively, and immediately prior to (d 0) and following (d 1, 2, 3, 6, 30, and 60) an intramammary (**IM**) challenge with *Escherichia coli*. Values are expressed with a mean shift of 5. Different superscripts denote significance ($P < 0.05$) between treatments within day.

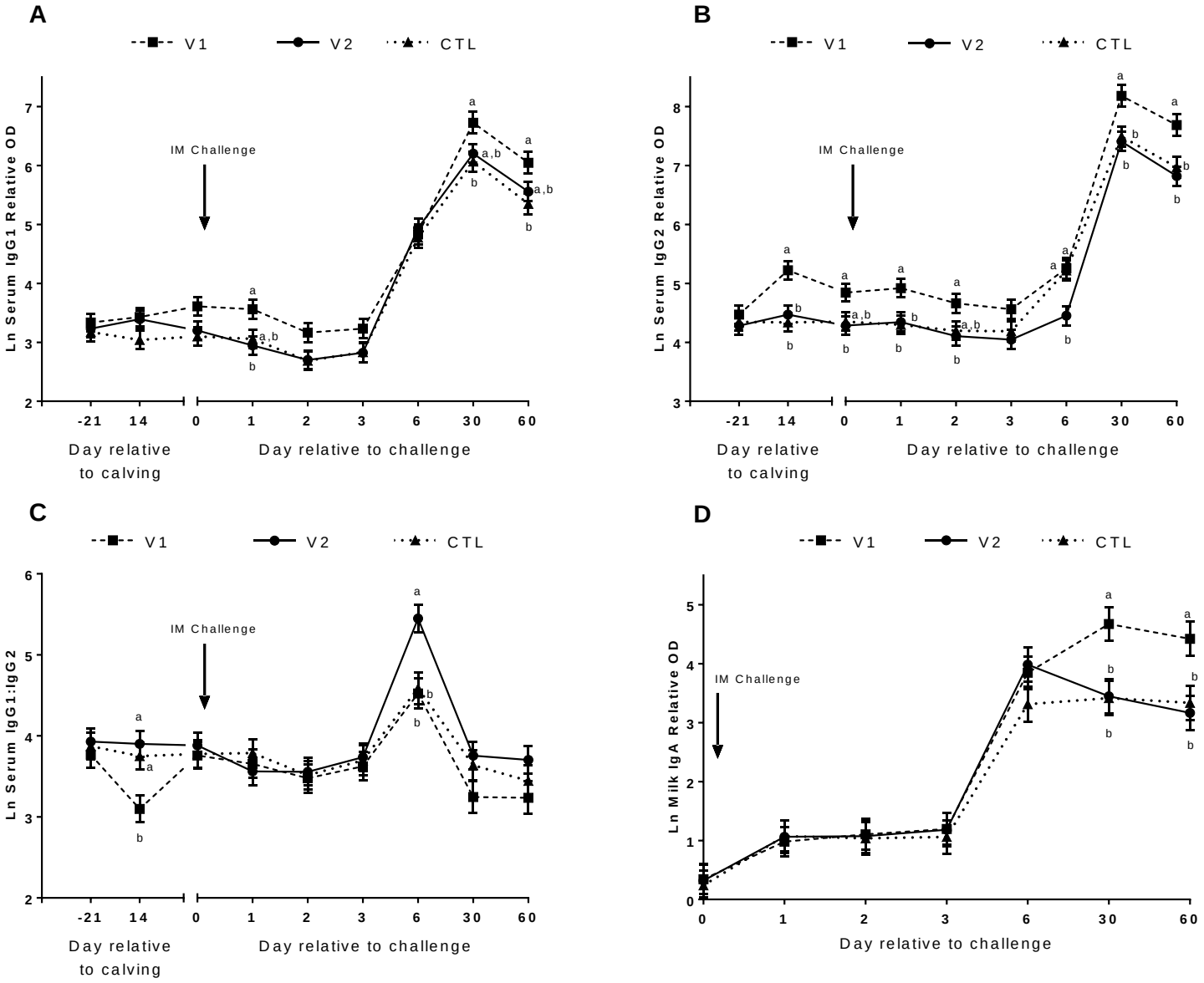

A
V1
V2
CTL
Ln Serum IgG1 Relative OD
IM Challenge
Day relative to calving
Day relative to challenge
B
Ln Serum IgG2 Relative OD
C
Ln Serum IgG1:IgG2
D
Ln Milk IgA Relative OD

Figure 2.7. Least squares means of the ln-transformed relative optical density of **A)** serum IgG1, **B)** serum IgG2, **C)** serum IgM, and **D)** serum IgA for primiparous and multiparous cows following the second and third vaccination given at d -21 and d 14 relative to calving, respectively, and immediately prior to (d 0) and following (d 1, 2, 3, 6, 30 and 60) intramammary (**IM**) challenge with *Escherichia coli*. Values are expressed with a mean shift of 5. Different superscripts denote $P < 0.05$ between parity within day.

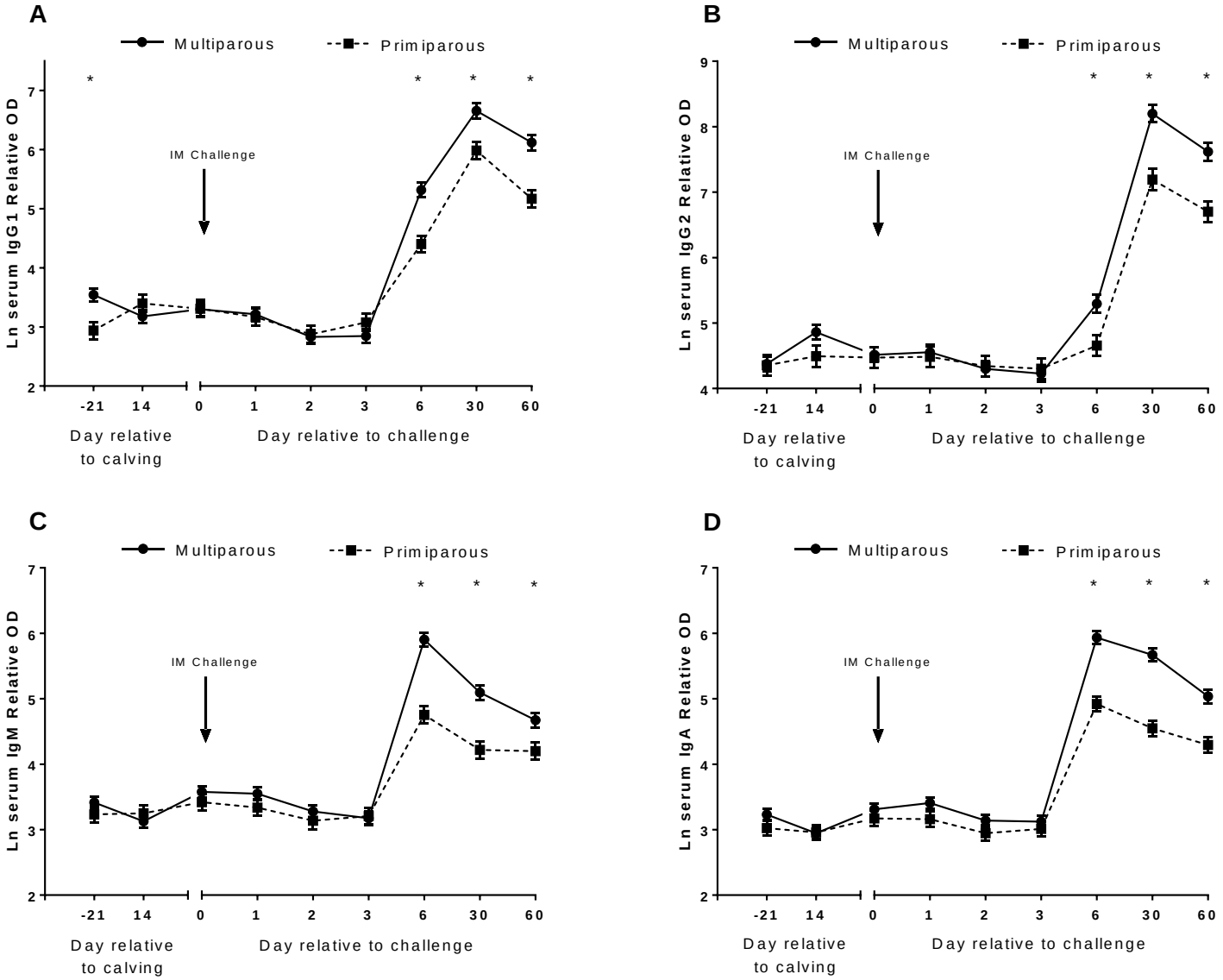
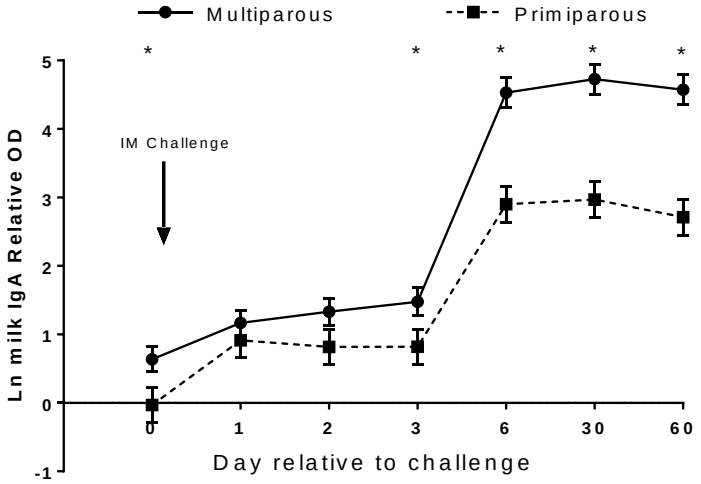

A
Multiparous
Primiparous
Ln serum IgG1 Relative OD
IM Challenge
Day relative to calving
Day relative to challenge
B
Ln serum IgG2 Relative OD
C
Ln serum IgM Relative OD
D
Ln serum IgA Relative OD

Figure 2.8. Least squares means of the ln-transformed relative optical density of milk IgA for primiparous and multiparous cows immediately prior to (d 0) and following (d 1, 2, 3, 6, 30 and 60) intramammary (**IM**) challenge with Escherichia coli. Values are expressed with a mean shift of 5. Different superscripts denote $P < 0.05$ between parity within day.



CHAPTER 3. Identifying Gram-negative and Gram-positive clinical mastitis using daily milk component and activity sensor data

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Mastitis algorithms using sensor data by Steele. Our study presents an evaluation of models derived from milk component and activity data for the identification of clinical mastitis cases caused by different bacterial groups including Gram-negative bacteria, Gram-positive bacteria and cases with no pathogen isolated. Separation into these different infection types improved mastitis classification accuracy, compared with models with all clinical mastitis cases grouped together. On-farm sensor data may provide an additional screening tool for detecting mastitic cows, and informing mastitis management decisions.

ABSTRACT

Opportunities exist for automated animal health monitoring and early detection of diseases such as mastitis with greater adoption of precision technologies on-farm. Our objective was to evaluate time series changes in individual milk component or activity variables for all clinical mastitis (CM) cases, and for Gram-negative and Gram-positive pathogen types and cases with no pathogen isolated. We developed algorithms using a combination of milk and activity parameters for predicting each of these infection types. Milk and activity data were collated for the 14 d preceding a CM event (n = 170) and for controls (n = 166), matched for breed, parity, and days in milk (DIM). Explanatory variables in the univariate and multiple regression models were the slope change in milk parameters (milk yield, conductivity, somatic cell count (SCC), lactose %, protein %, and fat %) and activity parameters (steps, lying time, lying bout duration, and no. of lying bouts) over 7 d. Slopes were estimated using linear regression between d -7 and -5, d -7 and -4, d -7 and -3, d -7 and -2, and d -7 and -1, relative to CM detection, for all parameters. Univariate analyses determined significant slope ranges for explanatory variables against the 4 responses; all CM cases (ACM), Gram-negative (GN) cases, Gram-positive (GP) cases, and no pathogen isolated (NPI) cases. Next, all slope ranges were offered into the multivariate models for the same 4 responses using 3 different baselines; d -10, -7 and -3 relative to CM detection. In the univariate analysis, no explanatory variables were found to be significant indicators of ACM cases, whereas at least 1 parameter was significant for each of GN, GP and NPI models. Multiple regression models for GN, GP and NPI revealed greater accuracy (82 to 86%), compared with the ACM model (74%). Sensitivity for the GN model was greater for the baseline closest to the day of CM detection (d -3), whereas the opposite was observed for the GP and NPI model, where sensitivity was greater when the -10 d baseline was used. Our analyses demonstrated a greater detection accuracy when infection types were considered separately, rather than all cases of CM grouped together. Based on this screening

of relationships, milk and activity sensor data could be used to inform the detection of CM on-farm.

Keywords: sensor data, slope change, pathogen type

INTRODUCTION

Mastitis remains an important and costly disease of dairy cows, with adverse effects on animal wellbeing and the quality and quantity of milk produced. The decision to treat a case of clinical mastitis (**CM**) is typically made without knowledge of the underlying cause of the disease because current diagnostic tools are based on culture or PCR technologies, limiting their use for real-time decision-making. More than 130 bacterial species have been associated with causing bovine mastitis (Watts, 1988), but approximately 10 species, or groups of species, account for 95% of mastitis infections (Makovec and Ruegg, 2003). Most mastitis pathogens can be classified into Gram-positive (**GP**) and Gram-negative (**GN**) bacteria, based on their cell wall characteristics. Prompt differentiation of CM cases as either GN or GP is important and allows for more appropriate treatment and management approaches. Infections due to GP bacteria typically respond well to antibiotic therapy, though low cure rates have been reported for *Staphylococcus aureus* (Wilson et al., 1999; Barkema et al., 2006). Infections due to GN such as *Escherichia coli*, usually present as acute, short-term CM episodes, with some developing into severe, systemic infections. Some GN infections could be better supported through use of anti-inflammatory approaches, since the bacteria can be quickly cleared by the host immune system (Pyörälä et al., 1994), rendering antibiotics unnecessary. Furthermore, GN are not susceptible to most commonly used antimicrobial products (Lago et al., 2011).

Currently, no cow-side and real-time diagnostic tool is available to differentiate between CM cases caused by GN or GP. Up to 40% of CM cases in the US are attributed to GN, with a further 10 to 40% of cases yielding no pathogen when cultured in the laboratory (Roberson, 2003). Considering many producers treat most CM cases with antibiotic therapy, regardless of the causative pathogen, up to 80% of the lactating cow antibiotics used to treat clinical cases could be deemed unnecessary (Roberson, 2012). Antimicrobial use in livestock production

systems is under increasing scrutiny, and treatment of mastitis without prior knowledge of the pathogen could be considered imprudent.

Producers require accessible, comprehendible, and reliable tools to detect and diagnose CM to inform management decisions in their dairy herds. Use of precision technologies on-farm is increasing, providing producers with new approaches to closely monitor changes in cow performance and behavior (Rutten et al., 2013). The large amount of data being generated daily provides an opportunity to monitor individual animals, and may enable early detection of diseases such as mastitis. Most cow-side tests for detecting mastitis rely on changes in the milk, including somatic cell count (**SCC**), sodium and chloride ion concentrations, or abundance of enzymes relating to tissue damage (Kitchen, 1981). Daily milk yield or lactose, protein and fat components may also shift in the days prior to visual detection of a CM case, whilst remaining relatively stable over time in healthy cows (Quist et al., 2008; Forsback et al., 2010). Cow activity including steps and lying behavior also change prior to detection of CM (Yeiser, 2011; Stangaferro et al., 2016; King et al., 2018). Individually, these changes may not be specific enough to indicate CM, but in combination, the accuracy of mastitis detection systems could be improved. Earlier detection and differentiation of CM cases could lead to quicker recoveries for the cow, and reduced economic losses for the producer (Milner et al., 1997).

The severity of mastitis is often related to the causative pathogen; infections by GN tend to induce a more severe inflammatory response, potentially with systemic involvement, whereas many GP pathogens tend to cause a more mild, or delayed inflammatory response (Schukken et al., 2011). These differences are likely to be reflected in milking performance, milk components and behavioral parameters. The objective of this study was to evaluate whether time series changes in individual milk components or activity variables were predictors of CM events, when events were categorized by pathogen type (GN, GP, or no pathogen isolated (**NPI**)) compared with all CM cases grouped together (**ACM**). A combination of milk and

activity parameters were then used to develop separate multiple regression models to identify ACM, GN, GP, or NPI cases.

MATERIALS AND METHODS

Study Design

A dataset containing milk yield, quality, and composition, and activity parameters for the 14 d preceding CM detection by milkers was collated for 170 CM events and 166 matched controls (Tholen, 2012). All lactating cows from the Virginia Tech **(VT)** Dairy Center (n = 230; Blacksburg, VA) and the University of Florida **(UF)** Dairy Unit (n = 480; Gainesville, FL) were available for enrollment to a case-control observational study. Data were collected at VT for 12 mo beginning in March 2011, and at UF for 9 mo from June 2011 (Tholen, 2012). Cows were milked twice daily at 12 h intervals, through a double 8 herringbone parlor at VT, or a double-12 herringbone parlor at UF. The herd consisted of Holstein, Jersey and crossbred animals at VT and Holstein at UF. All animals were housed in freestall barns, with sawdust bedding at VT and sand bedding at UF. Bedding was changed twice weekly at both facilities.

Case and Control Definitions

A CM case was defined as a cow with CM in one quarter (i.e., abnormal milk with clots and/or flakes, with or without redness and swelling of the mammary gland), as identified by trained milking staff during pre-milking teat preparation. At the onset of CM, the case cow had to be $\geq$ 15 DIM and $\leq$ 291 DIM and free of clinical signs for at least 14 d prior to enrollment. Only the first case of CM per lactation per cow were included in the dataset. A control animal was defined as not having visible signs of CM in the current lactation, and was retrospectively assigned to each case based on DIM, breed, parity and farm. Once a cow was enrolled as a case animal, that cow was not eligible to serve as a control for the remainder of that lactation. A single cow could be used as a control for multiple cases. Because controls were retrospectively

assigned to cases, milk samples were not collected from these animals to confirm absence of subclinical mastitis.

Milk Sample Collection and Diagnosis

Upon detection of CM, a quarter milk sample was aseptically collected by farm personnel following NMC guidelines (Middleton et al., 2017). Samples were stored at -20°C until transportation to the Virginia Tech Mastitis and Immunology Laboratory (Blacksburg, VA). Samples from VT were transported on ice once weekly to the laboratory, and samples from UF were shipped once monthly, on ice in an insulated cooler for overnight delivery.

Bacteriological procedures were completed in accordance with NMC guidelines (Middleton et al., 2017). Briefly, 10 µL of milk was streaked on one quadrant of an esculin blood agar plate and 100 µL of milk was streaked on one half of a MacConkey agar plate. Both plates were incubated at 37°C for 48 h. Bacterial identification was confirmed by colony morphology and biochemical testing (Middleton et al., 2017). A sample was considered contaminated if 3 or more dissimilar colony types grew in culture (Middleton et al., 2017). Samples that did not isolate bacteria under aerobic culture were cultured anaerobically for the presence of *Mycoplasma* spp.

Each CM case was assigned to 1 of 4 groups depending on the type of pathogen isolated: 1) GP pathogens, which included *Staphylococcus aureus,* coagulase negative *Staphylococcus* spp., and *Streptococcus* spp.; 2) GN pathogens, which included *Klebsiella* spp., *Escherichia coli*, *Citrobacter* spp., *Enterobacte*r spp., *Serratia* spp., *Pseudomonas* spp., and *Proteus* spp.; 3) other pathogens, which included *Prototheca,* yeast, and unknown microorganisms; and 4) NPI, where no pathogens were isolated under aerobic or anaerobic culture. Cases with mixed isolations (i.e., more than one pathogen isolated) and samples considered as contaminated were excluded from the study.

Data Collection

The VT and UF dairy farms were both equipped with herd management software (AfiFarm©, Afimilk Ltd., Kibbutz Afikim, Israel) for data storage. Milk components (milk protein, fat, and lactose percentages) and SCC were determined at each milking at the cow level using an in-line milk analyzer (AfiLab, Afimilk Ltd., Kibbutz Afikim, Israel). This system has been demonstrated to have moderate correlations with milk fat, protein and lactose percentages determined by DHIA using the Bentley 2000 instrument (Kaniyamattam and De Vries, 2014). The AfiFarm interface typically presents SCC data in categorical terms, but for this study, continuous numerical SCC data was retrieved from the Afimilk Ltd. database. Milk yield and electrical conductivity were determined at the cow level at each milking (AfiMilk MPC, Afimilk Ltd., Kibbutz Afikim, Israel).

Activity data was collected using pedometers attached to a rear leg fetlock on each cow. At VT, the pedometers were capable of measuring cow activity, as steps per hour, as well as daily lying time, number of lying bouts, and lying bout duration (AfiPedometerPlus, Afimilk Ltd., Kibbutz Afikim, Israel). A lying bout was only recorded if the duration of inactivity was > 3 min. At UF, each cow had a pedometer that measured only steps per hour (AfiPedometer, Afimilk Ltd., Kibbutz Afikim, Israel). Measurement of lying time and activity by the AfiPedometerPlus system has been previously evaluated (Higginson et al., 2010; Borchers et al., 2016). Milk and activity data were transmitted at milking time to the herd management software.

Milk and activity data were extracted for the 14 d preceding and 14 d following a CM case for the clinical cow and for the same date period for the matched control cow. All data were collated into a database (Microsoft Excel, Microsoft Corporation, Redmond, WA), and matched with the relevant bacteriological data. For each cow, electrical conductivity and milk components were averaged for the 2 milking sessions within a day. Hourly steps and lying bout

duration were averaged across all measurements for a 24 h period. Milk yield for the 2 sessions, and lying time and the number of lying bouts measured in a 24 h period, were summed to provide daily measurements. Somatic cell count was averaged across the 2 sessions within a day, and was log transformed (logSCC).

Determination of Changes in Variables Prior to Clinical Mastitis Detection

Ten explanatory variables were *a priori* considered for testing. These variables included daily measurements of milk lactose, protein and fat percentages, milk yield, electrical conductivity, SCC, average activity, number of lying bouts, average lying bout duration and total lying time. The slopes of each explanatory variable were estimated for each cow using linear regression in R v. 3.4 (R Core Team, 2017), for days ranging between d -7 and d -5, -4, -3, -2 or -1 relative to CM detection (d 0). Slopes of explanatory variables, rather than absolute values, were used to identify changes within cow over time, using each cow as her own baseline. For example, the slope of lactose percent between d -7 and -1 was estimated by linear regression, using values at d -7, -6, -5, -4, -3, -2, and -1 to represent the overall change in lactose over that time, whereas the slope of lactose percent between d -7 and -5 used lactose percent at d -7, -6 and -5 to estimate the overall change within this day range. Cases that had missing data on any one of the days used to compute slopes were not used for the determination of slopes for that particular day range.

Univariate Models

Univariate logit models were fit for each combination of response and explanatory variables using all slope ranges for each explanatory variable. Four different response variables were tested: ACM, GN, GP, and NPI. For the ACM univariate models, the response variable was all CM cases regardless of the cause, including cases for which no pathogen was isolated. Cows identified with CM were assigned a code of 1, and all controls were assigned a code of 0. For the GN models, the response was CM caused by GN pathogens; these cases were assigned a

code of 1 while all other CM cases and controls were assigned a code of 0. Likewise, for the GP models, the response was CM caused by GP pathogens; these cases were assigned a code of 1 and all other cases and controls were assigned a code of 0. For the NPI model, the response variable was NPI; these cases were assigned a code of 1 and all other CM cases and controls were assigned a code of 0. For each response variable, a total of 50 univariate models were tested; one model for each of the 5 slope ranges for each of the 10 independent variables considered. In all models, farm was included as a random effect.

Models were compared using a 3-part procedure. First, the optimal slope range (d -7 to -5, -4, -3, -2, or -1) was identified for each explanatory variable/response variable combination. The ideal slope range was determined first by eliminating models with *P* values > 0.1. Of those models with *P* values ≤ 0.1, the corrected Akaike's Information Criterion (AICc) of the models was compared to evaluate the likelihood of the model, given the data. The model with the lowest AICc was selected as the optimal slope range for that independent/dependent variable combination. Next, an optimal cutoff was identified for each model. Defining an optimal cutoff was necessary because the response of interest (infected vs healthy) was binomial; however, the model predicts a continuous spectrum of values between 0 and 1. To identify a threshold prediction value above which an animal was considered to be infected, all possible thresholds between 0 and 1 were tested using a step of 0.01. For each combination, the number of correct identifications were determined, depicted as model accuracy. A correct identification occurred when the model prediction was above the threshold for a clinical case or when the model prediction was below the threshold for a non-case (a control or a case caused by a pathogen other than the response of interest for that particular model). The threshold value with the largest proportion of correct identifications (greatest accuracy) was used as the cut-off, which determined whether an animal was predicted as a case or non-case by the model. Finally, to evaluate how these models, when converted to a binary response, compared in terms of their

ability to identify infections, the sensitivity **(Se)** and specificity **(Sp)** were calculated for each model. These values were then compared across explanatory variables to determine the strength of the relationships between the different explanatory variables and the responses.

Multiple Regression Models

Separate multiple regression models were derived with the same response variables of interest as the univariate models: ACM, GN, GP and NPI. Independent variables considered for inclusion in these models were: DIM, parity, breed, body weight, and the slope variables for milk and activity changes described previously. Mixed-effect logit models were used to relate response and explanatory variables. Farm was included as a random effect. Following procedures outlined in Roman-Garcia et al. (2016) and White et al. (2016), backwards stepwise elimination was used to derive the models where all variables remaining were $P \leq 0.1$. Variance inflation factor (**VIF**) was used to check for multicollinearity of explanatory variables remaining in the model after backwards elimination. Any explanatory variable with VIF > 5 was removed from the model.

Multivariate models for the 4 infection types were derived using 3 different baselines. Firstly, all parameters for all slope ranges using d -7 as the baseline (as in the univariate models) were offered into the model and backwards elimination was used to reach the final model. Next, separate multivariate models were developed using d -3 and d -10 as baselines to elucidate whether changes occurring closer to day of CM detection or more than 1 week prior to detection were important for the different pathogen types. For the models using d -3 as the baseline, slopes were generated using linear regression for each variable between d -3 and -2, -1 or 0. When d -10 was used as the baseline, slopes were generated between d -10 and -8, -7, -6, -5, -4 or -3 relative to CM detection. All multiple regression models were evaluated using a similar procedure to that described for the univariate models. First, an optimal cut-off was identified by testing all possible thresholds between 0 and 1 using a step of 0.01. For each combination,

the number of correct identifications were identified, and the threshold value was defined based on the greatest accuracy. Models were then evaluated by estimating the Se and Sp of each model. In the pathogen type models, the accuracy was weighted towards Sp, because the number of non-cases used in model derivation was greater than the number of cases.

RESULTS AND DISCUSSION

Data collected in a case-control study (Tholen, 2012) provided a unique dataset for analyzing changes in milk and activity parameters, prior to a CM case with known bacteriology. Studies comparing the physiological changes that precede a natural CM event are lacking, due to the difficulty in predicting when a clinical case will occur, and the logistics of identifying the causative pathogen. The increasing availability of precision technologies provides greater opportunities to measure milk and activity parameters for each cow at every milking, and to identify deviations from normal, which may be indicative of disease. Further, we proposed to categorize CM cases into groups based on pathogen type, to investigate whether changes occurring prior to CM detection were different for CM cases caused by GN bacteria, GP bacteria, or for NPI cases.

Data Summary

Complete data was obtained for a total of 336 animals (170 cases and 166 controls) for the 7 d preceding detection of a CM case (Table 3.1). Of the CM case animals, 67 (39%) were from VT and 103 (61%) were from UF, and for the control cows, 74 (45%) were from VT and 92 (55%) from UF. Numbers of cases and controls used in analyses differed by farm due to underlying incidence of CM on each farm, and exclusion of cases or controls with insufficient data records. Most cows were Holstein (n = 255), with the remainder being Jersey (n = 29) or mixed breed (n = 52), and 74% of animals were multiparous (n = 246). When categorized by bacterial group, most CM cases had no pathogen isolated (n = 90), followed by GN (n = 37),

GP (n = 35) and other pathogens (n = 8). The majority of GP pathogen isolations were *Streptococcus* spp. (57%) whilst *E. coli* (46%) was the most frequent GN pathogen isolated.

Univariate Models

Analysis of the 10 explanatory variables (slopes of protein %, lactose %, fat %, logSCC, milk yield, conductivity, activity, lying bouts, lying time, and lying bout duration) in univariate models revealed no significant indicators of CM when all cases were considered together (Table 3.2). Classification into GN, GP, and NPI, demonstrated some significant indicators of CM caused by these pathogen types.

For CM caused by GN pathogens, changes in step activity, lying time, conductivity and lactose correlated significantly with instances of CM. An increase in lying time between d -7 and -1 provided the greatest accuracy (72%) for CM caused by GN pathogens, with a Se of 52% and Sp of 77%. The next most accurate activity predictor was an increase in step activity, between d -7 and -5 relative to CM detection (Se = 82%, Sp = 59%, accuracy = 62%). Increased lying time is an indicator of sickness behavior in cows (Fogsgaard et al., 2015), supporting our finding of more time spent lying over the 7 d preceding clinical diagnosis. One study demonstrated reduced lying time at d -2 and -1 relative to naturally occurring CM, compared with uninfected control animals, which might be indicative of udder pain causing the cow to lay less (Yeiser, 2011). Although our data suggest an increase in step activity over d -7 to -5 relative to CM diagnosis, other studies have shown the opposite trend when comparing mastitic cows to healthy cows in the 5 d preceding CM diagnosis. Stangaferro et al. (2016) reported reduced activity in cows from 5 d before diagnosis of *E. coli* CM compared with healthy cows. Similarly, mastitic cows had reduced activity compared to healthy counterparts in the 3 d before diagnosis of CM caused by any pathogen (King et al., 2018). Reduced activity closer to CM detection makes biological sense, especially if the cow is spending more time lying. Using a cow as her own baseline is a useful approach because lying and activity behavior are highly

variable among cows. However, activity alone is not a specific indicator of CM, as changes can be associated with several other diseases (Edwards and Tozer, 2004) and with estrus (Kiddy, 1977).

The important milk parameters in the GN univariate analysis were an increase in lactose percent between d -7 and -2 and a reduction in conductivity between d -7 and -4 relative to CM detection. Sensitivity and Sp estimates for the lactose explanatory variable were 73% and 68%, and for conductivity, 83% and 61%, respectively. This finding of increased lactose percent and decreased conductivity contrasts with our understanding of the biological processes occurring in the mammary gland around CM, where lactose percent declines, and conductivity increases for mastitic cows (reviewed by Kitchen, 1981). King et al. (2018) reported greater maximum conductivity (highest quarter-level conductivity) in mastitic cows compared with non-diseased cows from 14 d before CM diagnosis. Mastitic cows also deviated from baseline levels of conductivity 12 d before CM diagnosis (King et al., 2018). In our study, milk constituents were measured at the cow level so changes occurring within one infected gland were diluted by other quarters. Nevertheless, the opposite conductivity effect seen here was unexpected. When d -3 (relative to CM detection) was used as the baseline, lactose declined between d -3 and -1, and -3 and 0, and the conductivity slope increased over the same day ranges (data not shown). Although these reflect the expected changes in milk around CM, these were not significant indicators of GN CM.

An increase in lying time between d -7 and -5 relative to diagnosis was the only significant individual indicator of CM caused by GP pathogens (Table 3.2). This model correctly identified 65% of all cases and controls, with a Se of 73% and Sp of 64%. Fewer parameters were found to be significant indicators of GP compared with GN. In the study by Stangaferro et al. (2016), the changes in activity were less pronounced for GP CM than for GN CM when comparing infected and healthy cows. Mastitis is typically considered an acute health disorder (King et

al., 2018); however, some cases of mastitis are chronic in nature (e.g. *Staph. aureus*), or may exist in subclinical form until cure, or until developing into CM (Schukken et al., 2011). In such cases, it is difficult to represent a cow's baseline because deviations from "normal" may be already occurring 7 d before clinical signs are detected. The use of an earlier baseline (e.g. 10 d prior to CM detection) resulted in more important parameters for detecting GP. There was a tendency for lactose percent, conductivity, lying time and milk yield changes between d -10 and -3, relative to CM detection, to be associated with GP (data not shown).

Reduced milk fat percentage between d -7 and -5, and reduced step activity between d -7 and -3, relative to CM detection, were significant indicators of NPI cases (Table 3.2). Of these models, the change in activity had the greatest accuracy (61%), with a Se of 77% and Sp of 55%. A culture-negative result from a truly clinical milk sample can occur for many reasons. Bacterial concentrations may be lower than the detection limit in culture, through either low shedding levels or death of bacteria during inappropriate storage conditions. Alternatively, bacteria may be growth-inhibited due to unsuitable media, culture conditions, or the presence of antibiotics (Middleton et al., 2017). For GN infections, signs of inflammation can persist after the bacteria have been eliminated by the immune system, leading to no pathogen being isolated from a clinically mastitic milk sample (Pyörälä et al., 1994). These NPI cases are thought to be more like GN cases for this reason. However, our findings suggest that NPI cases are unique, because there were differences in the significant parameters and direction of the relationship. The current study had 90 CM cases out of 170 with no pathogen isolated in culture, at a rate of 53%. The proportion of culture-negative cases was greater in CM samples from UF (67%) compared with VT (31%), which may be at least partially explained by the increased shipping distance for the samples from UF. Overall, the rate of culture-negative samples collected from CM cases in this study is greater than that reported in literature (10-40%; Taponen et al., 2009; Roberson, 2003).

Multiple Regression Models

Three different baselines (d -10, -7 and -3 relative to CM detection) were used to develop models for the 4 responses of interest. All but one of the models had some explanatory variables remaining in the model with $P < 0.1$ and VIF < 5. For the ACM cases model (Table 3.3), the best model performance was achieved using the d -10 baseline (reflecting the changes occurring between d -10 and -3, relative to CM detection). These parameters included a change in conductivity, lactose percent and SCC between d -10 and -7 and d -10 and -8, as well as protein percent between d -10 and -8, and number of lying bouts between d -10 and -3, relative to CM detection. This model correctly identified 74% of cases and controls, with a Se of 73% and Sp of 75%. Lactose and protein percentages were important across all baselines tested, indicating that changes in these parameters are consistent in CM, regardless of the causative agent. Lactose is the main osmotic regulator of milk with changes during mastitis well documented (Kitchen, 1981; Auldist et al., 1995; Pyörälä, 2003). Lactose percent declines, due to reduced synthesis by mammary epithelial cells, as well as leakage into interstitial fluid as the tight junction integrity becomes compromised (Kitchen, 1981). Milk proteins decline due to reduced synthesis and degradation by bacterial products, whilst serum proteins increase due to tight junction leakiness (Auldist et al., 1995), with the overall change in protein percentage dependent on the degree of damage sustained by the mammary epithelium. With greater technology uptake on modern dairy farms, daily measurement of lactose and protein percentages is becoming more common, enabling incorporation of these components into mastitis detection models.

For the GN model, changes in fat and lactose percent remained in models for all 3 baselines, and the change in activity was important for d -10 and -7 baselines (Table 3.4). The most accurate model for predicting both cases and controls was the d -10 model, with 82% accuracy, a Se of 41%, and Sp of 88%. However, the Se was greater for the model incorporating data

from the 3 d prior to CM detection (73%), but Sp was less (74%) than for the d -10 baseline. Model Se increased as the baseline moved closer to the day of CM diagnosis. The d -3 baseline may better capture the drastic changes occurring immediately prior to CM caused by GN pathogens, because these typically cause acute infections with rapid induction and regression (Schukken et al., 2011).

The most accurate GP model also used d -10 as the baseline (Table 3.5). Explanatory variables in this model included breed, and changes in activity (d -10 to -4), number of lying bouts (d -10 to -3), lying bout duration (d -10 to -8), and conductivity (d -10 to -7 and d -10 to -8). The accuracy was 86%, with a Se of 82% and Sp of 87%. The d -7 baseline was the next best at identifying GP, with 80% accuracy, 73% Se and 81% Sp. In contrast to the GN model, Se declined for GP models as the baseline neared the CM diagnosis. The GP model was able to detect twice as many clinical cases at d -10 than the GN model at the same baseline (Se 81% vs 41%), indicating that the d -10 baseline is more suitable for detecting CM caused by GP pathogens. This baseline represents a longer period of time series changes, which is sensible because GP infections are often of longer duration and may persist in subclinical form prior to clinical presentation of mastitis (Schukken et al., 2011).

The NPI model with the greatest case and non-case classification accuracy was the d -3 baseline, which included breed, and changes in lying bout duration and fat percentage between d -3 to -2 relative to CM diagnosis (Table 3.6). This model correctly identified 84% of cases and controls, with 62% Se and 88% Sp. The next best NPI model used d-10 as the baseline, which included breed, DIM, and changes in protein, lactose, fat, conductivity, activity and number of lying bouts. This had the best Se (94%) of all NPI models, and 77% Sp. The improved Se for detecting NPI cases at d -10 relative to diagnosis suggests some differences between these cases and GN cases, which had greater Se in the GN models closer to clinical

diagnosis. This finding supports our univariate findings of NPI cases being dissimilar to the other infection types.

Across the 4 response variables, more than 80% of cases and controls were correctly identified by the best GN model, the best NPI model, and all GP models, whereas the best ACM model had 75% accuracy, meaning that at least 25% were misclassified using the ACM models. Accuracy represents the correct classification rate of both cases and non-cases. The ratio of cases to non-cases was balanced for the ACM model, so Se and Sp were equally weighted for the calculation of accuracy. However, for the Gram-specific models, a smaller proportion of cases were available, meaning that the accuracy calculation was weighted more towards model Sp and the correct classification of non-cases, which included controls and cases caused by pathogens other than the response pathogen of interest. Therefore, consideration of Se and Sp together is more appropriate for the GN, GP and NPI models. When considering both Se and Sp, the GP model at d -10 baseline was the best performing model for detecting GP cases of CM, with a Se of 82% and Sp of 87%. These test characteristics are similar to those reported in the literature. In a review by Rutten et al. (2013), 37 sensor systems for automated mastitis detection were evaluated. Reported Se ranged from 55% to 89% and Sp ranged from 56 to 99%. However, none of these systems met the ISO/FDIS 20966 requirements of 80% Se with 99% Sp (Hogeveen et al., 2010). A Sp below 99% will result in many false alerts, but some producers may accept lower levels of Sp to create attention lists as a screening tool to assist mastitis detection (Kamphuis et al., 2008).

Data Challenges, Study Limitations, and Opportunities for Future Work

Our analyses included data relating to 170 cases of CM and 166 controls. Originally, the dataset contained 268 cases of CM and an equal number of controls (Tholen, 2012), but incomplete data in the period prior to CM detection meant that not all cases could be included. Our study used 4 different sensor systems to measure 10 milk and activity parameters and body weight.

Missing data is common in sensor systems due to errors in automatic data reading or entry in the parlor. For simplicity, and to represent real data collected using on-farm technologies, no data manipulation techniques were implemented in this study to make up for the missing data. One potential approach to deal with missing data is to generate the missing value by taking 95% of the previous day's value and 5% of the herds average for the current day. Additionally, using values from several consecutive days to create a rolling mean for the baseline value could have reduced the impact of the missing data.

Sensor systems such as the Afimilk technologies provide information at each milking, or more regularly for activity data, but we combined measurements into daily averages or sums for each variable. Improvements may be made in the detection accuracy by using session data, similar to the approach used by Jensen et al. (2016), where morning and evening session data were modelled separately. This was outside of the scope of the current study but may be considered for future work. The Afimilk meter and analyzer units provide cow composite measures of milk yield, conductivity, SCC category, and fat, protein and lactose percent. However, the effect of CM in one quarter is diluted by other uninfected quarters. Quarter-based measurements could improve predictions by detecting more pronounced changes at the quarter level. This approach is commonly used in automated milking systems, but cannot be applied in our analysis which used data from conventional farms. Further, our findings are limited to farms that have adopted technologies for measuring cow performance and behavior.

A simplistic modeling approach was used in this study, where slope changes in milk and activity variables were estimated by linear regression and modelled using logistic regression to estimate the log probability of being a case. This approach was selected over more sophisticated techniques to retain some biological understanding of the changes occurring before detection of CM. We included non-sensor information such as DIM, breed, and parity in our multiple regression models. Breed was important for indicating GP, and both breed and DIM were

important for NPI cases. We did not have previous CM history and somatic cell count available for inclusion into analyses, but previous studies have demonstrated improvements in predictive performance of algorithms for detecting mastitis when this information is incorporated into models (Steeneveld et al., 2010; Jensen et al., 2016).

In this study, model performance was evaluated internally, using the model training dataset, and we recognize the potential bias of this approach. Given the small number of cases in this dataset, it was not feasible to split the data into a training and validation dataset. An independent evaluation of multiple regression models derived in this study is underway to investigate the predictive ability of these models using an independent test dataset.

CONCLUSIONS

Individual activity or milk parameters were important for the categorized infection types, GN, GP, and NPI, whereas no parameters were found to be significant in the univariate analyses for ACM. Using a combination of milk and activity measures in multiple regression models, we demonstrated better model performance for CM categorized by pathogen type. Sensitivity was greater for detecting GN when the baseline was closer to the day of diagnosis (d -3), whereas the Se was greatest for detecting GP and NPI when data up to 10 d before clinical diagnosis were included in models. Further evaluation of these models using independent data is necessary to support our findings. Based on our analyses, changes in cow performance and behavior that precede a CM case differ for GN, GP and NPI cases. On-farm sensor data may be useful as an additional screening tool for detecting cows with CM, and informing mastitis management decisions.

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Table 3.1. Number of cases, split into pathogen type and bacteriological outcome, and controls, in the model training dataset, categorized by farm: **VT** = Virginia Tech Dairy Center, **UF** = University of Florida Dairy Unit.

	Farm		Total
Bacteriological outcome	**VT**	**UF**	
Gram-positive pathogens:			
Staphylococcus aureus	5	3	8
CNS	0	7	7
Streptococcus spp.	11	9	20
Total Gram-positive	16	19	35
Gram-negative pathogens:			
Klebsiella spp.	12	0	12
Escherichia coli	11	6	17
Citrobacter spp.	2	2	4
Enterobacter spp.	0	1	1
Serratia spp.	1	0	1
Unidentified Gram-negative	1	1	2
Total Gram-negative	27	10	37
Other pathogens:			
Prototheca spp.	0	3	3
Unknown microorganisms	1	1	2
Yeast	2	1	3
Total Other pathogens	3	5	8
No Pathogen Isolated	21	69	90
Total CM cases	67	103	170
Total controls	74	92	166
Total cases and controls	141	195	336

Table 3.2. Univariate models for each combination of response (all clinical mastitis cases (**ACM**), Gram-negative cases (**GN**), Gram-positive cases (**GP**), and cases with no pathogen isolated (**NPI**)) and explanatory variable at the slope range that had the lowest *P* value using -7 d relative to clinical mastitis detection as the baseline. For each model, the number of cases, intercept and slope estimates (± SE), and the accuracy, sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff are presented. Models with $P \leq 0.05$ are bolded. Models for all slope ranges are presented in Supplementary Table 7.1, 7.2, 7.3 and 7.4.

Response	Explanatory Variable	Slope Range	N. cases	Intercept (± SE)	Slope Estimate (± SE)	*P* value	Cutoff	Accuracy (%)	Se (%)	Sp (%)
ACM	Fat %	d -7 to -3	147	0.01 ± 0.12	-1.12 ± 0.70	0.11	0.50	55.3%	62.6%	48.0%
	Lactose %	d -7 to -5	129	0.01 ± 0.13	-1.32 ± 1.79	0.46	0.52	54.5%	20.9%	88.2%
	Protein %	d -7 to -1	157	0.03 ± 0.11	-3.27 ± 2.97	0.27	0.51	52.9%	49.0%	56.9%
	Conductivity	d -7 to -1	157	0.05 ± 0.12	-0.79 ± 0.59	0.18	0.52	55.8%	36.3%	75.8%
	Milk yield	d -7 to -1	157	0.06 ± 0.12	0.09 ± 0.08	0.27	0.46	53.5%	95.5%	10.5%
	LogSCC	d -7 to -4	143	0.04 ± 0.12	-0.26 ± 0.28	0.34	0.56	51.1%	4.2%	99.3%
	Activity	d -7 to -1	157	0.01 ± 0.12	-0.03 ± 0.02	0.13	0.54	53.2%	12.7%	94.8%
	Lying time	d -7 to -1	54	-0.15 ± 0.19	0.01 ± 0.01	0.17	0.49	61.2%	40.7%	79.0%
	Lying bouts	d -7 to -4	52	-0.13 ± 0.19	0.10 ± 0.12	0.42	0.49	57.1%	26.9%	83.3%
	Lying bout duration	d -7 to -1	54	-0.16 ± 0.19	0.05 ± 0.06	0.43	0.49	58.6%	27.8%	85.5%
GN	Fat %	d -7 to -5	33	-2.10 ± 0.57	0.94 ± 0.71	0.19	0.16	61.9%	75.8%	59.8%
	Lactose %	**d -7 to -2**	**37**	**-2.08 ± 0.52**	**8.57 ± 4.39**	**0.05**	**0.16**	**68.4%**	**73.0%**	**67.8%**
	Protein %	d -7 to -5	33	-2.09 ± 0.55	3.12 ± 2.10	0.14	0.13	60.3%	78.8%	57.6%
	Conductivity	**d -7 to -4**	**35**	**-2.15 ± 0.56**	**-1.48 ± 0.69**	**0.03**	**0.11**	**63.3%**	**82.9%**	**60.5%**
	Milk yield	d -7 to -3	35	-2.14 ± 0.56	0.12 ± 0.09	0.20	0.08	61.3%	82.9%	58.4%
	LogSCC	d -7 to -3	35	-2.12 ± 0.57	-0.30 ± 0.55	0.59	0.16	62.2%	77.1%	60.2%
	Activity	**d -7 to -5**	**33**	**-2.17 ± 0.60**	**0.03 ± 0.01**	**0.01**	**0.12**	**62.3%**	**81.8%**	**59.4%**
	Lying time	**d -7 to -1**	**23**	**-1.48 ± 0.25**	**0.02 ± 0.01**	**0.05**	**0.22**	**72.4%**	**52.2%**	**77.4%**
	Lying bouts	d -7 to -5	22	-1.40 ± 0.24	-0.13 ± 0.11	0.26	0.18	46.4%	81.8%	37.5%
	Lying bout duration	d -7 to -5	22	-1.40 ± 0.24	0.03 ± 0.03	0.31	0.17	37.3%	95.5%	22.7%
GP	Fat %	d -7 to -2	31	-2.18 ± 0.19	-1.10 ± 1.56	0.48	0.10	42.9%	64.5%	40.4%
	Lactose %	d -7 to -3	30	-2.19 ± 0.20	-3.94 ± 4.19	0.35	0.11	72.9%	36.7%	77.0%

	Protein %	d -7 to -3	30	-2.20 ± 0.20	4.27 ± 3.87	0.27	0.12	79.7%	23.3%	86.2%
	Conductivity	d -7 to -1	31	-2.18 ± 0.19	-1.27 ± 0.91	0.16	0.11	75.2%	32.3%	79.9%
	Milk yield	d -7 to -5	28	-2.13 ± 0.21	0.12 ± 0.08	0.15	0.12	73.9%	39.3%	78.2%
	LogSCC	d -7 to -5	28	-2.10 ± 0.20	-0.29 ± 0.37	0.42	0.13	83.5%	14.3%	92.1%
	Activity	d -7 to -5	28	-2.15 ± 0.21	-0.01 ± 0.01	0.10	0.11	71.2%	42.9%	74.7%
	Lying time	**d -7 to -5**	**11**	**-2.42 ± 0.38**	**0.01 ± 0.01**	**0.03**	**0.10**	**64.5%**	**72.7%**	**63.6%**
	Lying bouts	d -7 to -5	11	-2.27 ± 0.34	0.17 ± 0.14	0.20	0.10	57.3%	63.6%	56.6%
	Lying bout duration	d -7 to -2	12	-2.22 ± 0.32	0.05 ±0.07	0.48	0.12	82.8%	33.3%	88.5%
NPI	**Fat %**	**d -7 to -5**	**64**	**-1.22 ± 0.39**	**-1.49 ± 0.60**	**0.01**	**0.19**	**56.8%**	**85.9%**	**47.2%**
	Lactose %	d -7 to -2	81	-1.15 ± 0.44	-5.14 ± 3.52	0.14	0.22	58.8%	76.8%	52.3%
	Protein %	d -7 to -3	77	-1.15 ± 0.43	-2.71 ± 2.63	0.30	0.29	59.8%	74.0%	54.7%
	Conductivity	d -7 to -4	74	-1.18 ± 0.41	0.77 ± 0.44	0.08	0.31	65.7%	62.2%	67.0%
	Milk yield	d -7 to -5	64	-1.18 ± 0.38	-0.08 ± 0.06	0.19	0.29	64.6%	65.6%	64.2%
	LogSCC	d -7 to -3	77	-1.15 ± 0.43	-0.16 ± 0.35	0.65	0.18	59.1%	77.9%	52.3%
	Activity	**d -7 to -3**	**77**	**-1.19 ± 0.42**	**-0.03 ± 0.01**	**0.02**	**0.27**	**60.6%**	**76.6%**	**54.9%**
	Lying time	d -7 to -5	16	-1.79 ± 0.28	-0.00 ± 0.00	0.39	0.13	39.1%	87.5%	30.9%
	Lying bouts	d -7 to -5	16	-1.82 ± 0.28	0.15 ± 0.12	0.20	0.18	80.9%	31.3%	89.4%
	Lying bout duration	d -7 to -2	17	-1.73 ± 0.26	-0.05 ± 0.08	0.47	0.16	67.2%	41.2%	71.7%

Table 3.3. The final multivariate regression models for all clinical mastitis cases grouped together using 3 different baselines (d -10, d -7, and d -3, relative to clinical mastitis detection). For each model, the slope estimate (± SE) for the intercept and significant explanatory variables are presented at the cutoff where the percentage of cases and controls correctly identified (accuracy) are maximized, with the model sensitivity (**Se**) and specificity (**Sp**) at this cutoff.

Base-line	**Model**	**Slope estimate (± SE)**	***P* value**	**Cases (n)**	**Cutoff**	**Accuracy (%)**	**Se (%)**	**Sp (%)**
d -10				51	0.48	74.1	72.5	75.4
	Intercept	0.0 ± 0.2	0.89					
	Lying bouts; d -10 to -3	-0.9 ± 0.4	0.02					
	Conductivity; d -10 to -7	-5.1 ± 1.7	< 0.01					
	Conductivity; d -10 to -8	3.6 ± 1.2	< 0.02					
	Lactose %; d -10 to -7	-24.9 ± 8.6	< 0.01					
	Lactose %; d -10 to -8	17.9 ± 5.9	< 0.01					
	Protein %; d -10 to -8	9.1 ± 3.2	< 0.01					
	LogSCC; d -10 to -7	-2.9 ± 1.0	< 0.01					
	LogSCC; d -10 to -8	1.6 ± 0.7	0.02					
d -7				51	0.37	66.4	88.2	47.5
	Intercept	-0.1 ± 0.2	0.74					
	Lying time; d -7 to -1	0.0 ± 0.0	0.10					
	Conductivity; d -7 to -5	-1.2 ± 0.5	0.03					
	Lactose %; d -7 to -2	21.5 ± 8.8	0.01					
	Protein %; d -7 to -1	12.5 ± 8.4	0.14					
d -3				46	0.4	64.6	76.1	54.7
	Intercept	-0.2 ± 0.2	0.41					
	Lying time; d -3 to -1	0.0 ± 0.0	0.05					
	Lactose %; d -3 to -2	3.9 ± 1.9	0.04					
	Protein %; d -2 to -1	5.2 ± 2.6	0.04					
	LogSCC; d -3 to -2	0.6 ± 0.3	0.02					

Table 3.4. The final multivariate regression models for Gram-negative clinical mastitis cases using 3 different baselines (d -10, d -7, and d -3 relative to clinical mastitis detection). For each model, the slope estimate (± SE) for the intercept and significant explanatory variables are presented at the cutoff where the percentage of cases and controls correctly identified (accuracy) are maximized, with the model sensitivity (**Se**) and specificity (**Sp**) at this cutoff.

Base -line	Model	Slope estimate (± SE)	*P* value	Cases (n)	Cutoff	Accuracy (%)	Se (%)	Sp (%)
d -10				32	0.16	82.1	40.6	87.5
	Intercept	-2.2 ± 0.2	< 0.01					
	Activity; d -10 to -3	0.1 ± 0.0	0.01					
	Activity; d -10 to -6	-0.0 ± 0.0	0.01					
	Fat %; d -10 to -3	-3.2 ± 1.3	0.02					
	Lactose %; d -10 to -5	-9.8 ± 5.9	0.09					
d -7				33	0.14	71.2	63.6	72.3
	Intercept	-2.0 ± 0.2	< 0.01					
	Fat %; d -7 to -2	4.9 ± 2.0	0.02					
	Lactose %; d -7 to -2	12.3 ± 6.4	0.05					
	Activity; d -7 to -5	0.0 ± 0.0	0.02					
d -3				21	0.24	73.3	71.4	73.8
	Intercept	-1.6 ± 0.3	< 0.01					
	Lying bout duration; d -3 to 0	0.1 ± 0.0	0.08					
	Fat %; d -3 to -2	1.6 ± 0.7	0.02					
	Lactose %; d -3 to -2	3.5 ± 2.0	0.08					

Table 3.5. The final multivariate regression models for Gram-positive clinical mastitis cases using 3 different baselines (d -10, d -7, and d -3 relative to clinical mastitis detection). For each model, the slope estimate (± SE) for the intercept and significant explanatory variables are presented at the cutoff where the percentage of cases and controls correctly identified (accuracy) are maximized, with the model sensitivity (**Se**) and specificity (**Sp**) at this cutoff.

Base-line	**Model**	**Slope estimate (± SE)**	***P* value**	**Cases (n)**	**Cutoff**	**Accuracy (%)**	**Se (%)**	**Sp (%)**
d -10				11	0.18	86.1	81.8	86.6
	Intercept	-3.9 ± 0.8	< 0.01					
	Breed	2.0 ± 0.8	0.02					
	Activity; d -10 to -4	-0.2 ± 0.6	0.02					
	Lying bouts; d -10 to -3	-1.9 ± 0.8	0.02					
	Lying bout duration; d -10 to -8	-0.1 ± 0.1	0.03					
	Conductivity; d -10 to -7	-7.7 ± 2.9	0.01					
	Conductivity; d -10 to -8	3.7 ± 1.8	0.04					
d -7				11	0.12	80.0	72.7	80.8
	Intercept	-3.3 ± 0.6	< 0.01					
	Breed	1.9 ± 0.8	0.01					
	Lying time; d -7 to -5	0.0 ± 0.0	0.08					
	Lactose %; d -7 to -5	-14.0 ± 6.9	0.04					
d -3				19	0.19	85.5	47.4	89.4
	Intercept	-2.9 ± 0.4	< 0.01					
	Breed	1.7 ± 0.6	< 0.01					
	Activity; d -3 to 0	0.0 ± 0.0	0.04					
	Protein %; d -3 to -2	-4.0 ± 1.7	0.02					

Table 3.6. The final multivariate regression models for clinical mastitis cases with no pathogen isolated using 3 different baselines (d -10, d -7, and d -3 relative to clinical mastitis detection). For each model, the slope estimate (± SE) for the intercept and significant explanatory variables are presented at the cutoff where the percentage of cases and controls correctly identified (accuracy) are maximized, with the model sensitivity (**Se**) and specificity (**Sp**) at this cutoff.

Base -line	**Model**	**Slope estimate (± SE)**	***P* value**	**Cases (n)**	**Cutoff**	**Accuracy (%)**	**Se (%)**	**Sp (%)**
d -10				16	0.13	79.6	93.8	77.2
	Intercept	-5.3 ± 1.3	< 0.01					
	DIM	0.0 ± 0.0	0.05					
	Breed	2.3 ± 0.8	0.01					
	Protein %; d -10 to -3	-73.3 ± 36.8	0.05					
	Protein %; d -10 to -4	73.6 ± 31.0	0.02					
	Lactose %; d -10 to -5	-69.3 ± 23.0	< 0.01					
	Fat %; d -10 to -5	-9.5 ± 4.9	0.06					
	Conductivity; d -10 to -5	-7.7 ± 3.8	0.04					
	Activity; d -10 to -7	0.1 ± 0.0	0.09					
	Lactose %; d -10 to -8	10.8 ± 6.5	0.10					
	Lying bouts; d -10 to -7	-0.9 ± 0.4	0.03					
	Lying bouts; d -10 to -8	0.4 ± 0.2	0.08					
d -7				64[1]	0.24	59.1	76.6	53.4
	Intercept	-1.1 ± 0.2	< 0.01					
	Breed	-2.3 ± 1.0	0.03					
	Activity; d -7 to -1	-0.1 ± 0.0	0.04					
	Milk yield; d -7 to -4	0.2 ± 0.1	0.08					
	Milk yield; d -7 to -5	-0.2 ± 0.1	0.02					
d -3				13	0.20	84.2	61.5	87.5
	Intercept	-2.8 ± 0.5	< 0.01					
	Breed	1.2 ± 0.7	0.06					
	Lying bout duration; d -3 to -2	-0.0 ± 0.0	0.08					
	Fat %; d -3 to -2	-2.0 ± 0.9	0.03					

[1]Number of cases are greater for d -7 baseline because cases from both farms are included due to the lack of significance of activity parameters that were specific to VT only.

CHAPTER 4. Independent evaluation of mastitis detection algorithms for identifying Gram-negative and Gram-positive clinical mastitis using daily milk component and activity sensor data

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Mastitis detection model evaluation by Steele. Our study independently evaluated previously derived models to determine whether clinical mastitis detection using on-farm sensor data was better when clinical mastitis cases were categorized into Gram-negative cases, Gram-positive cases, or cases with no pathogen isolated. Model performance estimates were less favorable during independent evaluation, with the best model for Gram-negative pathogens reaching 62% accuracy. Larger datasets with more clinical mastitis cases are necessary to develop models with greater accuracy.

ABSTRACT

Increasing adoption of precision technologies on-farm has provided a platform for automated animal health monitoring, and early detection of diseases such as mastitis. Previously, we developed models that incorporated milk component and cow activity data to identify clinical mastitis (CM) caused by different bacterial groups. Our objective in this study was to independently evaluate 12 multiple regression models for their ability to identify CM caused by: 1) all CM cases (ACM), 2) Gram-negative (GN) pathogens, 3) Gram-positive (GP) pathogens and 4) cases with no pathogen isolated (NPI). Each CM model used 3 different baselines: d -10, d -7, and d -3, relative to CM detection, to compare time series data over different ranges. Models were derived using the change in milk (milk yield, fat %, protein %, lactose %, SCC, conductivity) and activity (steps/h, daily lying time, number of lying bouts, and lying bout duration) parameters, over 10 d prior to CM detection. The training dataset contained milk and activity data relating to 170 CM cases and 166 controls, matched for days in milk, breed and parity. The test dataset included daily milk and activity data for 230 cows over 20 mo, with 102 CM cases. For ACM, data between d -3 and d 0 resulted in the optimal performance, with sensitivity of 56% and specificity of 52%. Likewise, data in the 3 d prior to CM detection produced the best performing model for detecting GN, with 62% sensitivity and specificity. The sensitivity of detecting GP was <32% for all baselines, with specificity ranging from 62% to 82%. Categorizing by pathogen type improved GN detection, compared with ACM, but the sensitivity of detecting CM caused by GP and NPI was <50% for all 3 baselines. The study was limited by the number of CM cases available for model training and independent evaluation, and by the differences in infection distribution between the training and test dataset. Future model development should utilize larger datasets that better reflect the prevalence of mastitis pathogens for which the models are to be applied in practice. On-farm sensor data can

be useful for animal health monitoring, but model improvements are necessary before implementation as management tools on-farm.

Keywords: model evaluation, mastitis pathogens

INTRODUCTION

Mastitis continues to be an important disease of dairy cows, affecting approximately 25% of cows each year in the US (USDA, 2016). The average cost of a clinical mastitis **(CM)** case was estimated at $179 (Bar et al., 2008), increasing to $444 for a case in the first 30 days of lactation (Rollin et al., 2015). Currently, detection of CM on most conventional farms relies on the visual assessment of milk for abnormalities. Diagnosis of the pathogen causing the underlying infection requires laboratory methods such as culture, which usually takes a minimum of 3 d for a diagnosis to be reported to the producer (Middleton et al., 2017). As a result, most producers treat CM without knowledge of the bacterial pathogen, which contravenes responsible antimicrobial stewardship. Infections caused by Gram-negative (**GN**) pathogens, such as *Escherichia coli* and *Klebsiella spp.*, and cases with no pathogen isolated, are not considered to be candidates for broad-spectrum antimicrobial treatment, which may represent 50 to 80% of CM cases, depending on the herd (Roberson, 2012).

Better diagnostic tools are needed on-farm to support treatment decisions, and reduce unnecessary antimicrobial use. On-farm culture (OFC) systems are available in the US, which reduce the time for a diagnosis to 1 d (Royster et al., 2014; Ganda et al., 2016). These systems have been demonstrated to reduce AMU by more than 50%, without impacting bacteriological cure outcomes (Lago et al., 2011). However, OFC systems were regarded as cost-effective only in herds with GN pathogens as the dominant cause of CM (Down et al., 2017). Alternatively, increasing adoption of precision technologies on conventional dairy farms enables automated monitoring of individual cows for diseases such as mastitis. Automated milking systems already rely on sensor technologies for indicating cows suspected of having mastitis. A review of sensor technologies measuring individual cow parameters that relate to mastitis (e.g. milk electrical conductivity, milk color or presence of relevant mastitic enzymes in milk) demonstrated that most available mastitis detection systems provide information based on

sensor data to inform about the cow's health status (Rutten et al., 2013). Although these detection systems provide useful information, improvements in detection accuracy are necessary, and most tools lack the ability to guide decision making (Rutten et al., 2013). Advanced technologies on-farm now enables more parameters relating to milk composition and cow behavior to be measured on a daily basis. When used in combination with other sensor data measuring SCC, electrical conductivity or milk yield, mastitis detection systems could be improved (Jensen et al., 2016). Furthermore, it may be possible to use this sensor data to indicate pathogen type (Kamphuis et al., 2011), as mastitis caused by Gram-positive (**GP**) and GN bacteria differ in terms of pathogenesis, which may be differentially expressed as alterations in milk yield, milk components or cow behavior in the lead up to a CM event.

Previously, we hypothesized that models classifying CM cases into those caused by GN bacteria, GP bacteria or cases with no pathogen isolated (**NPI**) would improve the detection accuracy, when compared with all CM cases grouped together (**ACM**; Chapter 3). We derived models that utilized time series changes in milk and activity parameters collected on-farm. Initial evaluation of these models, based on the training dataset, demonstrated greater detection accuracy for the models that categorized CM by pathogen type (82 to 86%), compared with the ACM model (74%; Chapter 3). However, the previously derived models require independent evaluation, to determine whether they are repeatable under an independent setting. The objective of this study was to evaluate the previously derived models for detecting 1) ACM, 2) GN, 3) GP, and 4) NPI, using time series data at 3 different baselines, relative to CM detection (d -10, d -7, and d -3). For the external evaluation of mastitis detection models, a test dataset was retrospectively collated, which contained daily milk and activity data for 230 cows, collected over a 20 mo period. Within the test dataset, a total of 102 CM cases existed.

MATERIALS AND METHODS

Development of multiple regression models

As previously described (Chapter 3), data were collected in a case-control study (170 CM cases, 166 matched controls; Tholen, 2012), from 2 research farms: Virginia Tech Dairy Center (Blacksburg, VA) and the University of Florida Dairy Unit (Gainesville, FL). Data related to the 14 d preceding and 14 d following a case of CM or a control selected retrospectively. Variables in the dataset included daily measures of milk yield and electrical conductivity (AfiMilk MPC, Afimilk Ltd., Kibbutz Afikim, Israel), milk fat %, protein %, lactose %, SCC (AfiLab, Afimilk Ltd.) and daily activity measures, including average steps/h (AfiPedometer, Afimilk Ltd.), lying time, lying bout duration and number of lying bouts (AfiPedometerPlus, Afimilk Ltd.). Multiple regression models were derived as previously described (Chapter 3) for 4 responses of interest: 1) ACM, 2) GN, 3) GP, and 4) NPI**.** Slope changes in the daily milk and activity measures were used as explanatory variables, and were estimated using linear regression in R v. 3.4 (R Core Team, 2017) over a 10 d period preceding CM detection. Following preliminary findings of inconclusive results for the NPI model, this was excluded from further evaluations. Following preliminary findings of inconclusive results for the NPI model, this was excluded from further evaluations. Following preliminary findings of inconclusive results for the NPI model, this was excluded from further evaluations. The explanatory variables offered into each model for ACM, GN, GP, and NPI included all slopes (within the aforementioned range for each specific baseline) of all 10 milk and activity variables, along with breed, parity, body weight and DIM. Infection was treated as a binomial response (infected vs not infected) and farm was included as a random effect. Backwards stepwise elimination was used to derive final models which included variables that remained significant ($P \leq 0.05$) or tended to be significant ($P \leq 0.1$). The optimal cutoff was identified by testing all possible thresholds between 0 and 1, using a step of 0.01, and the threshold value was defined based on the largest proportion of correct identifications of cases and controls.

Using this cutoff, models were evaluated internally using the model training dataset (Chapter 3).

Test dataset

An independent test dataset was created using data collected retrospectively from cows at the Virginia Tech Dairy Center between August 2015 and April 2017. Milk and activity data were collected as described earlier and were retrieved electronically from Afimilk Ltd. (Kibbutz Afikim, Israel). The milk and activity data were combined with cow data including parity, breed, DIM, and body weight. Clinical mastitis records including cow identification number, date of CM, bacterial pathogen type or species isolated and treatment records were collated from 3 sources: paper records in the farm office, records entered into PC Dart (Dairy Records Management System, Raleigh, NC), and culture results of milk samples processed at the Virginia-Maryland College of Veterinary Medicine (VMCVM) culture laboratory (Blacksburg, VA). The farm policy was to collect a milk sample from the affected quarter(s) of a cow upon detection of CM. Farm managers rely on milking staff to collect a sample and report CM events on a sheet in the parlor. Milk samples were stored at -20°C until collection by the herd veterinarian, or delivery to the VMCVM culture laboratory by farm staff. Milk samples were cultured for bacteriology following NMC guidelines (Middleton et al., 2017). Culture results were recorded in the laboratory, and reported back to farm managers, who recorded this information in PC Dart, and on the paper CM records. In some instances, milk samples may have been collected for reasons other than CM (e.g., high SCC from the monthly DHIA test). Additionally, some true cases of CM may have been recorded, but for some reason a sample was not collected. For our study, a set of criteria were developed to determine CM cases based on the data recorded at the time of the event. If a quarter was recorded as clinically mastitic either in PC Dart, or in the farm office records, this was considered as a CM case and was coupled with the culture results from the VMCVM laboratory. However, if no milk sample

was collected, this potential CM event could not be included as a CM case, as the necessary bacterial culture information was not available. If 2 or more quarters were sampled from a cow on the same day, it was only considered a CM event if antibiotic treatments were given, assuming that on most farms, a large proportion of cows with CM are treated upon detection (Oliveira and Ruegg, 2014), and cows with subclinical mastitis are not treated, adhering to industry recommendations (Ruegg, 2017).

A total of 102 CM cases were recorded in 20 mo (Table 4.1). Cases of CM were classified into bacterial groups based on the predominant pathogen that grew in culture. The groups included GP pathogens (n = 56), GN pathogens (n = 23), other pathogens (n = 1), NPI (n = 11), contaminated samples (n = 7) and mixed infections (n = 4). A case was considered a mixed infection when pathogens from different group classifications were isolated at the same time, from either the same quarter, or from different quarters of the same cow if at more than 1 quarter presented with CM. If 2 bacterial pathogens were isolated from the same quarter or cow, but were from the same group classification, the case was kept in analyses. The CM cases that were classified as contaminated or mixed were excluded as cases because bacteriology results were inconclusive. A CM case that occurred within 10 DIM was also excluded because models required milk component and production data in the 3 to 10 d prior to CM detection. Additionally, a second recurrence of CM within 14 d of a prior case was not considered a separate case, but multiple cases from the same cow that occurred > 14 d apart were included.

Model Evaluation

Models were evaluated by comparing observed and predicted cases of CM and calculating sensitivity (**Se**), specificity (**Sp**), and the proportion of correctly identified cases and non-cases (accuracy). The observed CM cases were defined as a cow identified with CM on a given day (e.g. d 0) and were the gold standard, true positives. The non-cases were cows that did not have CM on any given day, and were considered the gold standard, true negatives. Data for the 14

d preceding and 14 d following CM were not used in the model evaluation for non-cases. A predicted case of CM was a cow with a predicted value that exceeded a certain cutoff on any given day, whereas a non-case had a predicted value below that cutoff. The initial cutoff tested was the threshold value demonstrated to be optimal during internal evaluation of each model (Chapter 3). Next, a new cutoff was determined using the approach described earlier, where all possible thresholds between 0 and 1 were tested using a step of 0.01. This second optimal cutoff resulted in the largest proportion of correct identifications of cases and non-cases, and was defined as the retrained cutoff. Sensitivity and Sp of each model were estimated for both the original and retrained cutoffs.

RESULTS AND DISCUSSION

Data summary

A total of 102 CM cases observed over 20 mo were available for model evaluation. Of these CM cases, 56 were GP (55%), 23 were GN (23%), and 11 were NPI cases (11%). The contamination rate was 7% of all samples collected (Table 4.1). The majority of GP pathogens were *Streptococcus* spp. (n = 31), followed by coagulase-negative staphylococci (n = 20), and most GN CM cases were caused by *E. coli* (n = 14). This is a shift in predominant pathogens isolated from the same herd, 3 years prior, where GN pathogens were isolated from 40% of CM cases, GP pathogens from 24% and NPI represented 31% of CM cases (Chapter 3). The CM incidence in the test dataset was 5.1 cases/mo, slightly less than the incidence rate of 5.6 cases/mo in the training dataset (Chapter 3). However, the herd was moved to a new facility at the beginning of the current study's data collection period, which may account for the lower incidence and change in proportion of GP and GN pathogens isolated from CM cases. Herd managers anecdotally reported that fewer cases of mastitis were occurring, especially GN cases, possibly due to a change in bedding material from sawdust to sand.

Model performance

Of the 3 baselines used, the ACM model with the most optimal performance (59% Se, 44% Sp) for detecting CM was the d -3 baseline, which included changes in lying time and protein % between d -3 and -1 relative to CM, and changes in lactose % and SCC between d -3 and -2 (Table 4.2). When the retrained cutoff was used, the optimal Se and Sp still occurred at the d -3 baseline, with similar Se (56%) and Sp (52%). Low Se combined with high Sp was observed at the other baselines (d -10 and -7, relative to CM). In contrast, our previous evaluation using the training dataset demonstrated that the "best" model was the d -10 baseline, incorporating data between d -10 and -3 relative to CM detection, with greater Se and Sp estimates of 73% and 75%, respectively (Chapter 3). The appropriate baseline for ACM depends on the predominant pathogens causing CM. In the test dataset, GP pathogens were the leading cause of CM (55% of all CM cases), whereas the training dataset consisted of similar proportions of GN and GP pathogens (20 and 21% of all CM cases), but the majority were cases with NPI (53%; Chapter 3).

Numerous studies have reported the use of one or multiple sensors for predicting CM, with the given output as a mastitis alert, a probability or degree of infection, or classification into subclinical, clinical or healthy. The reported detection performance varied considerably, with Se between 32% and 100% and Sp between 56% and 99.8% (reviewed by Hogeveen et al., 2010; Rutten et al., 2013). A wide range of approaches for predicting or classifying CM have been used, including artificial neural networks, fuzzy logic, discriminant analysis, decision tree induction, naïve Bayesian networks, moving thresholds, and time series analysis, using typically electrical conductivity as the main sensor input, but also milk yield, temperature, flow rates, color and SCC (reviewed by Hogeveen et al., 2010). A recent study utilized more sensor inputs than many previous studies, including milk composition and non-sensor information in a multi-variate, dynamic linear model with a naïve Bayesian classifier to produce a probability of mastitis (Jensen et al., 2016). Using this approach, the estimated Sp was 81%, at a Se set at

80%. The relatively low estimates of Se and Sp for detecting ACM in the current study, and the differences between CM caused by different pathogen types, make a good case for categorization of pathogen type to improve CM detection using sensor data.

For the detection of GN CM, the best performing model used the d -3 baseline, with both Se and Sp of 62% at the original cutoff value, and Se of 54% and Sp of 86% using the retrained cutoff (Table 4.2). Change in fat and lactose percent between d -3 and -2, relative to CM, and lying bout duration between d -3 and the day of CM detection (d 0), were indicators of GN CM in the final model. Both Se and Sp were highest at the d -3 baseline, whereas other baselines using data ≥ 7 d prior to CM detection had Se < 39% and Sp between 60% and 80% depending on the cutoff used. These results agree with our previous evaluation using the training dataset, which identified the best baseline to be d -3 (Se 71%, Sp 74%). It was expected that more pronounced milk and activity changes occurring during a case of GN CM would be observed closer to the appearance of visual signs of CM (d 0) since these infections are typically acute with a sudden onset (Schukken et al., 2011; Pyörälä et al., 1994). Model performance could be improved by use of more CM cases due to GN, as just 21 cases were used to develop the d -3 GN model and 13 cases from the test dataset were used to independently evaluate the model. A lack of cases is a common limitation of studies that have developed models to differentiate CM by Gram-status (Kamphuis et al., 2011).

The model with the highest Se for detecting GP used the d -7 baseline, with 32% Se and 62% Sp achieved at the original cutoff, and 23% Se and 85% Sp at the retrained cutoff (Table 4.2). This model included breed, and changes in lying time and lactose percent between d -7 and -5 relative to CM. However, these Se estimates are less than those reported in our internal evaluation (Se = 82%, Sp = 87; Chapter 3). Specificity reached 98%, but at this level of Sp, only 2 GP CM cases (6%) were identified. We grouped both major and minor GP pathogens together, but it is important to note that differences exist in their pathogenesis. Infections

caused by *Staph. aureus* and environmental streptococci caused more damage to mammary epithelial cells than minor pathogens such as coagulase-negative staphylococci (**CNS**), causing more changes in milk composition and SCC (Schukken et al., 2011). Kamphuis and others (2011) excluded CNS from the GP pathogen group in a similar study classifying CM events by Gram-status; however, accuracy was similar to that reported in this study. We included these pathogens because they represented a significant proportion of GP CM cases in the training (20%) and test datasets (36%). Although the number of cases in this study were not sufficient to further categorize GP pathogens into *Staphylococcus aureus, Streptococcus* spp. and minor pathogens such as CNS, this is one strategy that could improve model performance (Kamphuis et al., 2011: Steeneveld et al., 2009).

Cases with NPI were most often detected when the d -7 baseline was used, which resulted in 50% Se (Table 4.2). Specificity for this model was between 54 and 58%, depending on the cutoff used. Breed, and change in step activity between d -7 and -1, and change in milk yield between d -7 and -5 and d -7 and -4, were parameters remaining significant in this NPI model. Greater Sp was demonstrated in the d -3 model, (72% for the original cutoff, and 91% for the retrained cutoff), with a slight reduction in Se (40%). We previously reported better Se (94%) and Sp (77%) at the d -10 baseline (Chapter 3). The conflicting performance and baselines between studies is not surprising because our evaluation is based on a small number of NPI cases (n = 5 to 8), so these findings are limited, and require more data to draw any conclusions.

Our results suggest better performance of GN models compared with GP models, at least in terms of Se. Previous research indicates GN CM is easier to identify than GP CM when considering cow demeanour, milk appearance and extent of milk yield decline (White et al., 1986; Milne et al., 2003). Veterinarians assessing CM cases that were severe enough to warrant attention were able to predict coliform CM cases (36 coliform and 82 non-coliform cases) with a Se of 64% and Sp of 61%, but specific criteria used to make predictions were not described

(White et al., 1986). A logistic regression model with milk yield and cow demeanour as predictors had a Se of 28% and Sp of 96% for detecting GN CM when applied to a test dataset (Milne et al., 2003). The model was developed using 573 CM cases, of which 153 were caused by GN pathogens and 420 were caused by GP pathogens (Milne et al., 2003). Gram-negative cases of CM had greater odds of having watery milk, systemic signs of illness, and greater reductions in milk yield, compared with CM caused by GP pathogens, minor pathogens or cases with NPI (Milne et al., 2003). Assessment of CM cases by veterinarians is not normal procedure on-farm, especially for mild to moderate CM cases, so this kind of approach is not practical on most dairy farms, and does not remove the subjectivity of classifying cases of CM.

An automated approach to pathogen type CM identification could be used on a larger scale and, if accurate, would provide a better opportunity to inform treatment decisions. One study developed a decision tree, incorporating parameters relating to the range or increase in electrical conductivity at the quarter level, compared with previous milkings, or other quarters at the same milking (Kamphuis et al., 2011). The accuracy was high (91%) when evaluated on training data, but declined to 55% for test data, with authors suggesting a need for more CM cases for building the decision tree, or the integration of more, or improved, sensor and non-sensor information. In a large study across 274 herds with 3,833 CM cases, Steeneveld and others (2009) used naïve Bayesian networks to develop probability distributions for the causal pathogen and the Gram-status of a CM event, with 73% accuracy. Though our models fail to reach this level of accuracy, our findings highlight the potential for improved accuracy using data from various sensor inputs and different time ranges relative to CM detection for the different pathogen types. Further model development or consideration of different modelling approaches may lead to improved performance using the knowledge gained in this study.

Challenges with farm data

We collated records from multiple sources to increase the accuracy of recorded CM events, as these records formed the gold standard for model evaluation. A gold standard should best reflect what happens in reality, to limit the potential impact of incorrectly identified CM cases and non-cases on the development and validation of models (Rutten et al., 2013). We acknowledge that some CM cases will have been missed due to incomplete recording, failure to collect milk samples from truly clinically mastitic quarters or failure to obtain definitive results from culture, in the case of contaminated samples or mixed infections (n = 11). Alternatively, some cases may have been incorrectly interpreted as CM when milk samples were collected, and treatment was administered to cows that had subclinical infections. In general, treating subclinical infections is thought to be a rare occurrence (Ruegg, 2017). We recognize the limitations of retrospective data collection in forming the test dataset for independent model evaluation, but undertook methods to improve the accuracy of the gold standard where possible. On-farm recording of CM events is far from perfect, and protocols to optimize and improve the accuracy and consistency of CM recording have been proposed, but not yet adopted on most dairy farms (Wenz, 2018).

Another important factor in defining the gold standard and for comparison across studies is the time window used to describe an alert as successful, when associated with a CM event. In the current study, the identification of CM by the model was required on the same day that CM was observed, to provide a successful outcome. Other studies have used longer time windows, which allow for a slight mismatch in the timing of a CM event with the alert (Kamphuis et al., 2008). A time window of 48 h has been recommended as appropriate, because producers may perceive an alert more than 1 d before clinical signs to be a false alarm and lose trust in the system, and 1 d after CM is observed is likely too late (Hogeveen et al., 2010). Extension of the time window from 24 h to 48 h in this study might have improved model Se and Sp estimates; however, we did not want to overstate performance by using a wider time window.

Comparison of training and test datasets

To compare the internal and external evaluation, both the original and retrained cutoff Se and Sp were considered. In all models, Se was greater for internal evaluation of models, compared with the external evaluation in the current study. Overestimated Se in the previous work was a possibility due to the inherent bias of evaluating models using the data that was used to derive models; however, splitting the data into a model training and test dataset to cross-validate was not appropriate due to the limited abundance of CM cases. The finding that Se was overestimated initially was therefore, not a surprise in the current study. Retraining the cutoff generally reduced Se and increased Sp, because the retrained cutoff was at the threshold value where the largest number of correct identifications of cases and non-cases were made. The dataset used in our study contained many more records relating to non-cases than cases, so a greater weighting was automatically applied to Sp at the selected retrained cutoff.

The infection distribution of the datasets for internal and external evaluation were very different. The training dataset was built from a case-control study (Tholen, 2012), so approximately 50% of the records related to a CM case, whereas the other 50% were associated with controls (considered healthy non-cases; Chapter 3). A crude calculation puts the total number of records at < 10,000 in the model derivation dataset, because it contained a maximum of 29 d of data for each of the 166 controls and 170 cases, though only up to 10 d of data was used in deriving and evaluating models. Of the 170 CM cases, 37 were GN and 35 were GP CM, so assuming an average of 7 d of data for each CM case, 7% of the data within the training dataset was associated with GN CM and an additional 7% with GP CM cases, respectively. In stark contrast, the test dataset was collected retrospectively for an average of 230 milking cows over 690 d, without removing non-case data or matching cases with controls. As a result, this dataset reflected the typical level of CM seen in practice. For a dataset that contained upwards of 130,000 records, and just 102 cases of CM, < 0.5% of the records in that dataset relate to a

CM case. The differences between the 2 datasets highlights one challenge with using case-control data for model derivation if those models are intended for on-farm application.

CONCLUSIONS

Independent evaluation of the previously derived multiple regression models for detecting ACM, or categorized CM cases caused by GN, GP or NPI, identified that model performance was initially overestimated, and that models lacked repeatability. Retraining the cutoff based on the independent test dataset generally improved Sp estimates but for many models, the Se was limited. The best model for detecting GN CM used data in the 3 d preceding CM, in agreement with our previous work. This GN model's estimated performance exceeded that of the ACM model. However, the GP models were not better than the ACM model, at least when considering Se, which conflicts with the model evaluation based on training data. Model performance requires further improvement, which could be achieved with more CM cases caused by each pathogen type, or by application of models on data that are more similar to the infection distribution of the training dataset. On-farm sensor data provide a good resource for animal health monitoring; however, current models are not yet accurate enough as management tools on dairy farms.

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Table 4.1. Summary of pathogen type classifications and bacterial groups or species isolated from clinical mastitis cases in the test dataset.

Bacteriological outcome	**Total**
Streptococcus spp.	31
CNS	20
Staphylococcus aureus	5
Total Gram-positive	56
Escherichia coli	14
Klebsiella spp.	6
Unidentified Gram-negative	3
Total Gram-negative	23
Corynebacterium spp.	1
Total Other pathogens	1
No Pathogen Isolated	11
Contaminated sample	7
Mixed infection	4
Total	102

Table 4.2. The sensitivity (**Se**) and specificity (**Sp**) for detecting all clinical mastitis cases (**ACM**), Gram-negative clinical mastitis cases (**GN**) Gram-positive clinical mastitis cases (**GP**) and clinical mastitis cases with no pathogen isolated (**NPI**), using 3 different baselines (d -10, -7, and -3 relative to clinical mastitis detection) at the original cutoff (**OC**; from the model training dataset) and the retrained cutoff (**RC**; from the test dataset)

Model	Baseline	Cutoff used	Cutoff value	Cases (n)	Se (%)	Sp (%)
ACM	d -10	OC	0.48	51	45.1	48.0
		RC	0.99	51	19.6	87.3
	d -7	OC	0.37	51	57.1	39.9
		RC	0.98	49	16.3	92.6
	d -3	OC	0.40	46	58.8	43.9
		RC	0.46	34	55.9	51.5
GN	d -10	OC	0.16	32	38.9	70.6
		RC	0.19	18	38.9	76.4
	d -7	OC	0.14	33	38.9	59.5
		RC	0.29	18	33.3	81.4
	d -3	OC	0.24	21	61.5	61.5
		RC	0.63	13	53.8	86.4
GP	d -10	OC	0.18	11	18.8	82.2
		RC	0.92	32	6.3	98.0
	d -7	OC	0.12	11	31.8	62.1
		RC	0.76	44	22.7	85.4
	d -3	OC	0.19	19	27.3	75.4
		RC	0.49	44	18.2	91.6
NPI	d -10	OC	0.13	16	28.6	49.1
		RC	0.03	7	71.4	38.5
	d -7	OC	0.24	64	50.0	53.5
		RC	0.25	8	50.0	58.3
	d -3	OC	0.20	13	40.0	71.7
		RC	0.37	5	40.0	90.6

CHAPTER 5. Field evaluation of mastitis detection models for identifying Gram-negative and Gram-positive clinical mastitis

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Field evaluation of Gram-specific mastitis models by Steele. In this study, we developed 3 multiple regression models that used changes in individual cow milk and activity changes over 7 d to detect any case of clinical mastitis (CM), or CM due to either Gram-negative or Gram-positive pathogens. On-farm implementation of models on 2 herds for 5 mo demonstrated inadequate sensitivity ranging from 7% to 21%, but high specificity (> 99%). In the current state, our models provided little value in detecting CM or intramammary infection. Model training using more CM cases could improve detection performance, and assist in pathogen type differentiation to inform treatment decisions.

ABSTRACT

Improved detection of clinical mastitis (CM) on-farm and rapid diagnosis of the causative pathogen are necessary to reduce negative effects on animal welfare and milk quality, and for judicious antimicrobial use. The study objective was to develop and test 3 separate models for indicating any CM case (ACM), or CM caused specifically by Gram-negative (GN) or Gram-positive (GP) pathogens. The slopes of milk and activity variables from d -7 to d -5, -4, -3, -2 or -1, relative to CM detection, were estimated and offered into models, along with cow information including parity, days in milk, breed and body weight. Final models retained variables that were significant. Models were tested on 2 research farms (University of Kentucky [UKY]; Virginia Tech [VT]) for 5 mo, and evaluated for their ability to detect CM, or intramammary infection (IMI), defined as the presence of pathogens detected in culture. A total of 672 cows had foremilk sampled from each quarter to determine IMI status: 83 had a CM case, 478 were alerts by any 1 of the 3 models, and 111 cows were randomly selected for sampling. The sensitivity and specificity of detecting ACM was 21% and 99%, respectively. Only 1 in 5 CM cases had a successful ACM alert at VT, and 1 in 4 CM cases coincided with an ACM alert at UKY. Gram-specific models provided no advantage for CM detection over the ACM model, and no model had combined high sensitivity and specificity for detecting IMI. Implementation of the Gram-specific models derived at VT was not successful in detecting any GN or GP cases at UKY, though our study was limited by sample size. Based on current performance, these models provide little value as a mastitis detection system in a commercial farm setting. Training models with more CM cases could improve models for CM detection, and potentially provide a tool for differentiation of CM by Gram-status to inform treatment decisions.

Keywords: sensor data, pathogen type, clinical mastitis detection

INTRODUCTION

Mastitis in dairy cows is a common and costly disease affecting animal welfare and productivity, as well as milk quality. Mastitis is also a major contributor of antimicrobial use on dairy farms; a quarter of antibiotics are used for treating clinical mastitis (**CM**), and 70% to 80% are attributable to udder health in general (Pol and Ruegg, 2007; van Werven, 2013; Kuipers et al., 2016). Most cases of CM are treated without regard to the specific pathogen causing the intramammary infection (**IMI**), resulting in ill-advised antimicrobial treatment of CM cases caused by Gram-negative (**GN**) bacteria or no pathogen (up to 80% of CM cases; Roberson, 2012).

Early detection and rapid diagnosis of the etiological agent can lessen the impact of mastitis, and inform treatment decisions. Detection of CM on conventional farms relies on visual assessment of milk for abnormalities during pre-milking teat preparation, whereas in automated milking systems **(AMS)**, inbuilt sensors and algorithms are designed to alert producers to cows suspected of having mastitis (Hogeveen and Ouweltjes, 2003). Diagnosis of the causative pathogen for individual CM cases usually requires laboratory support for bacterial culture or molecular analysis. Recently, on-farm culture systems have become available to indicate the type of pathogen causing mastitis, though still taking 1 d for a result (Royster et al., 2014). As the dairy industry shifts towards more responsible use of antimicrobials, researchers continue to develop tools for timely CM detection, with many working towards producing diagnostic tools that can be implemented in real time, including interventions targeted to the pathogen type (Steeneveld et al., 2009; Kamphuis et al., 2008, 2011).

The performance of current mastitis detection systems for identifying CM has been reviewed extensively (Hogeveen et al., 2010; Rutten et al., 2013), and authors agree that while these systems are useful, improvements are necessary to reach the desired sensitivity **(Se)** of 80% and specificity **(Sp)** of 99% for use in AMS (Hogeveen et al., 2010). Improvements can be

made in the sensors themselves, or in the algorithms that process the sensor data. Using sensor data, few have attempted to categorize CM cases by Gram-status (Steeneveld et al., 2009; Kamphuis et al., 2011). Steeneveld and colleagues (2009) used a naïve Bayesian network to develop probability distributions for the Gram-status of 1,202 CM cases, with 73% accuracy. Inputs into the naïve Bayesian network included Gram-specific CM history, milk color, SCC, and whether the cow was sick or not. A smaller study of 140 CM cases described a decision tree incorporating various electrical conductivity and milk color measurements at the quarter level (Kamphuis et al., 2011). For predicting the Gram-status, the accuracy was 91%, based on the training dataset, but accuracy dropped to 55% for a test dataset.

Considering the physiological effects of mastitis on mammary gland function and milk composition (Kitchen, 1981), and the flow-on effects on cow demeanor or behavior (White et al., 1986; Yeiser, 2011), we reasoned that inclusion of more sensor inputs, representing milk composition and cow behavior, would improve CM detection, and possibly assist in indicating the Gram-status of the causative pathogen. Previously, we derived several multiple regression models using matched case-control data (Chapter 3), but implementation using real-farm data did not demonstrate improved performance of the Gram-specific models, compared with all CM cases grouped together (Chapter 4). Since herds differ with respect to the prevalence of CM and the predominant pathogens causing infection, a model developed on one herd or dataset might be limited in its predictive ability for a different herd (Hogeveen et al., 2010). The objective of this study was to generate multiple regression models from real-farm data containing daily milk and activity parameters, to detect all CM cases (**ACM**), or CM caused specifically by Gram-negative (**GN**) or Gram-positive (**GP**) pathogens. Models were tested for their ability to detect CM or IMI on the model training herd and a second independent herd. We hypothesized that detection performance would be improved when categorizing CM by Gram-status, compared with the ACM model.

MATERIALS AND METHODS

Model development

Multiple regression models were derived from a dataset containing 136,127 cow day records and 102 cases of CM observed in cows at the Virginia Tech Dairy Center (Blacksburg, VA) between August 2015 and April 2017 (described in detail in Chapter 4). Sensor data included daily measures of milk yield and electrical conductivity (AfiMilk MPC, Afimilk Ltd., Kibbutz Afikim, Israel), milk fat %, protein %, lactose % (AfiLab, Afimilk Ltd.) and average steps/h, lying time, lying bout duration and number of lying bouts (AfiPedometerPlus, Afimilk Ltd.). The milk and activity data were combined with non-sensor information including parity, days in milk (**DIM**), breed, and body weight. Separate models were developed for the following response variables: ACM, GN, and GP. The responses were binomial; non-mastitic or clinically mastitic for the ACM model, and CM caused by GN or GP for the Gram-specific models. Models were derived using the approach described in Chapter 3. Briefly, the slopes of each sensor variable (milk yield, conductivity, fat, protein, and lactose percent, steps/h, lying time, lying bout duration, and number of lying bouts) were estimated using linear regression in R v. 3.4 (R Core Team, 2017). For the slope calculation, the baseline day was d -7 for time series data, relative to CM detection, which was selected to utilize the changes in milk and activity data occurring in the week prior to CM for predictions. Slopes between d -7 and -5, -4, -3, -2, and -1, relative to the day of CM detection, were computed for each sensor variable, and all were offered into each of the 3 models, along with parity, DIM, breed and body weight. Backwards stepwise elimination was used to reach final models with remaining variables significant ($P \leq 0.05$) or tending to be significant ($P \leq 0.1$). The optimal cutoff for categorizing a cow as clinically mastitic or non-mastitic was identified by testing all possible thresholds between 0 and 1, using a step of 0.01. The threshold value selected had the greatest accuracy (proportion of correct identifications of cases and non-cases). At this selected cutoff, the 3

models were evaluated by comparing the observed cases of CM in the training dataset with the predicted CM cases, and calculating the Se and Sp of each model.

Herd descriptions

Models were tested on 2 research farms: Virginia Tech Kentland Dairy Center (**VT**; Blacksburg, VA) and the University of Kentucky Coldstream Dairy (**UKY**; Lexington, KY). Cows at the VT dairy (n = 230) were milked twice daily through a double-12 parallel parlor and were housed in sand-bedded freestalls. Cows at the UKY dairy (n = 110) were milked twice daily through 2 side-by-side double-2 parlors and were housed in a compost bedded pack facility. The study ran from March to August 2018.

Data editing

Raw data from VT were retrieved electronically once daily from the Afimilk Ltd. database. The raw data from UKY were stored in AfiFarm and uploaded automatically once daily to an internet-based file storage system (DropBox, DropBox Inc., San Francisco, CA). Raw data were session-based, so prior to generating model predictions each day, data were summarized into daily sums for milk yield, lying time, and number of lying bouts, and into daily averages for electrical conductivity, milk fat %, lactose %, protein %, steps/h and lying bout duration. Continuous SCC data were available for VT cows, but not for UKY cows; therefore, SCC was not used as an explanatory variable in model derivation.

Model predictions for generating a daily cow alert list

Following data editing, slopes were generated for each of the variables previously described, and those remaining in the final 3 models (Table 5.1) were used to generate 3 predicted values for each cow that had the preceding 7 d of data available. The ACM, GN and GP models contained the same parameters for the 2 farms, but the cutoff for UKY was reduced by a magnitude of 10 following preliminary investigations that demonstrated very few alerts when

the original cutoffs (optimized during model training data from VT) were used. For the ACM model, a mastitis alert was defined as a cow with a predicted value of ≥ 0.03 at VT and ≥ 0.003 at UKY. For the GN model, a mastitis alert occurred when a cow's predicted value was ≥ 0.02 at VT and ≥ 0.002 at UKY. A mastitis alert for the GP model was defined as a predicted value ≥ 0.05 at VT and ≥ 0.005 at UKY. For every day of the study period, a list of cows that exceeded the thresholds for any 1 of the 3 models was generated for each farm, prior to the afternoon milking. An alert by any 1 of the 3 models triggered the aseptic sampling of milk from all quarters of the cow at the afternoon milking on the day of the alert. An individual cow was sampled no more than once per week; e.g., if a cow alerted a second time within 7 d of the first alert, a second sample was not collected. However, if the same cow alerted > 7 d following the original alert, sampling was repeated for that cow. Prior to commencing the study, herd managers and milking staff on each farm agreed that the acceptable maximum for sampling on any given day would be 5 cows. In most cases, the milk samples were collected on the same day as an alert, but occasionally, milking staff would miss a cow, or research staff would delay sampling for an alerted cow if too many were scheduled for the same day. As such, a cow may have been sampled 1 to 6 d following the alert, potentially missing a transient IMI present on the day of the alert, but not detectable in the days following when a sample was taken. Although this could penalize model performance if the alert was wrongly labelled as a false negative, the models were developed to detect CM not transient IMI, so the impact on evaluating the model's ability to detect CM was considered negligible.

Observation of clinical mastitis

Milk was visually assessed for CM by trained milking staff at every milking, during pre-milking teat preparation at both farms. Teat preparation involved 30 s contact time with a teat disinfectant, and visual assessment of several streams of foremilk from each quarter before wiping each teat dry. Clinical mastitis was diagnosed cow-side as visibly abnormal milk with

clots, flakes or discoloration, with or without redness or swelling of the mammary gland. Upon detection of CM, all quarters of the affected cow were to be sampled before being milked or administered treatment, and the cow identification number, affected quarter, date, and any treatments were recorded on a form in the parlor. In addition, the sample tube from the affected quarter was marked with 'CM'. If a cow presented with CM in the same quarter again in the 7 d following the first clinical episode, a second sample was not collected. A CM event was defined as a new case if a second episode occurred > 14 d after the previous episode, even if the same pathogen was isolated from the same quarter (Hertl et al., 2011).

Random sampling

To assess the IMI status of cows that did not alert, a random sampling scheme was implemented alongside the sampling for alerted cows. For every 5 alerts generated on each farm, an additional cow was randomly selected for sampling. Cows in the milking herd for each farm were ordered numerically by their identification number, and assigned a number between 1 and the maximum number of cows being milked on the day a cow was randomly selected. A random number was then generated using the RANDBETWEEN function in Microsoft Excel (Microsoft Corporation, Redmond, WA). A new number was generated if the random cow selected also alerted that day, because the goal was to represent the level of IMI in cows that did not have a mastitis alert.

Milk sample collection and pathogen classification

Milk samples were collected from all functional quarters of cows that either alerted, were randomly selected or were observed with CM during the study period. Sample collection was undertaken mostly by milking staff, and, occasionally, by research staff at VT. Training was provided by research staff at the beginning of the study, and a visual representation of aseptic sampling techniques were displayed in the milking parlors as a reminder for existing staff and

a teaching resource for new staff. Foremilk was collected using aseptic technique following NMC guidelines (Middleton et al., 2017). The tubes were labelled with the cow identification number, date, farm, and quarter, and marked with "CM", if appropriate, and stored at -20 °C. Samples from VT were collected from the farm at least twice weekly and transported on ice to the Virginia Tech Mastitis and Immunology Laboratory (Blacksburg, VA). Samples from UKY were shipped once a month, on ice, in a Styrofoam cooler.

Bacteriological cultures were completed for all milk samples following NMC guidelines (Middleton et al., 2017). In brief, each quarter milk sample was streaked on a quadrant of esculin blood agar (10 µL) and a half of MacConkey agar (100 µL), and incubated for 48 h at 37°C. Growth was recorded at 24 h and 48 h. Bacterial identifications were confirmed by Gram staining, assessment of colony morphology and biochemical testing (Middleton et al., 2017). A sample was considered contaminated if ≥ 3 different species grew in culture (Middleton et al., 2017). All bacterial identifications were completed by the same person for consistency.

Cows were categorized into the following groups based on their quarter bacteriological outcomes from samples collected on the same day: GN pathogens, GP pathogens, other pathogens, no pathogen isolated, mixed infection, and an inconclusive result. Gram-negative pathogens included *Escherichia coli*, *Klebsiella* spp., *Serratia* spp., *Citrobacter* spp., *Enterobacter* spp., and unidentified GN organisms. Gram-positive pathogens included *Streptococcus* spp, *Staphylococcus aureus*, coagulase-negative staphylococci (**CNS**), and *Corynebacterium bovis*. A cow was classified as having no pathogen isolated if all 4 quarters had no growth in culture. A cow was categorized as GN if ≥ 1 quarter had a GN pathogen isolated, or if a different GN pathogen was identified in the same or another quarter of a cow, provided that no quarters from that cow had a GP or other pathogen isolated, and that the criteria for classifying a contaminated sample was not violated. Likewise, a cow was categorized as GP if ≥ 1 quarter had a GP pathogen isolated, or if multiple GP pathogens were

isolated from the same cow on the same day. The same rules applied to cows categorized into the other pathogen group. If a quarter was infected with a GP pathogen and the same quarter or a different quarter from the same cow was infected with a GN (or other) pathogen, the cow was classified as having a mixed infection. When a quarter was classified as contaminated, and occurred alongside another quarter that had a GN or GP pathogen isolated, the contaminated sample was overlooked, and the cow was categorized by the pathogen identified. However, if 1 or more quarters were contaminated, and the remaining samples from that cow were culture-negative, the result was inconclusive, because it was not certain whether the contaminated sample was from a quarter free of infection. A cow was also classified as inconclusive if no samples were collected from a cow that alerted or had CM.

Model evaluation

In the first evaluation, on-farm CM detection on a single day served as the gold standard for comparison against the 3 models. For the ACM model, a correct classification occurred when a case of CM was detected on the same day as an ACM alert for that cow (i.e., the predicted value exceeded the threshold), or on a day when a cow was free of CM with no ACM alert (i.e., the predicted value was below the threshold). A false positive occurred when there was an ACM alert, but no CM detected, and a false negative occurred when a cow had CM but did not have an ACM alert. For the Gram-specific models, a true positive was defined as CM caused by either GN or GP pathogens on the same day as a GN or GP alert, respectively. A true negative occurred for a cow with no CM caused by GN pathogens and no GN alert, or no GP CM with no GP alert. A false positive was defined when a GN or GP alert occurred when respective Gram-specific CM were not detected, and a false negative occurred when no Gram-specific alert coincided with the respective Gram-specific CM case. The Se and Sp were calculated along with the success rate **(SR)** and the false alert rate per 1000 cow milkings **(FAR1000)**, which are more useful measures for producers (Hogeveen et al., 2010). The SR is

the proportion of alerts that are likely to be correct, which is synonymous with the positive predictive value **(PPV)**. The FAR1000 depicts the number of false alerts that occur in 1000 cow milkings, emphasizing to producers the frequency of alerts that, when checked, prove to be a false alarm.

Although the models were designed to detect CM, subclinical infections also alter milk composition, so we also evaluated the ability of the 3 models to detect IMI. The gold standard was a cow with an IMI in any quarter with the respective pathogen of interest (ACM = any pathogen; GN = Gram-negative pathogen; GP = Gram-positive pathogen). Cows that alerted or were randomly sampled at VT and UKY were used in the evaluation, and any cow sampled for CM was excluded. Additionally, cows with inconclusive results from bacteriology could not be included, and for evaluating the GN and GP models specifically, mixed infections were excluded. A true positive occurred when a culture-positive result coincided with an ACM alert, or when a GN or GP pathogen was cultured from a cow that had a GN or GP alert, respectively. A true negative was a culture-negative result from a randomly sampled cow (no alert). A false positive was an ACM, GN or GP alerted cow that was IMI-negative for the respective pathogen, and a false negative was a randomly sampled cow that was IMI-positive (with the respective pathogen). The Se and Sp of detecting IMI, along with the PPV and negative predictive value **(NPV),** were estimated for each of the 3 models separately at each farm and for the 2 farms combined. Because we did not assess all cows at every milking for IMI, the FAR1000 could not be calculated.

RESULTS

Model parameters

The ACM model for detecting any CM case included parity, breed, and the changes in conductivity, milk fat % and milk yield between d -7 and -1 relative to CM, as well as the change in conductivity between d -7 and -4, and d -7 and -5, and milk yield between d -7 and

-5 (Table 5.1). At the cutoff where model performance was optimized on the training dataset, the Se was 66.1% and Sp was 68.8%. The GN model included parity, and slopes of activity (d -7 to -2), conductivity and milk yield (d -7 to -3), with estimated Se of 60% and Sp of 98.1%. The GP model was similar to the GN model in that it included changes in conductivity and milk yield between d -7 and -3 relative to CM, but had low Se (4.5%), with high Sp (100%).

Model performance for detecting and classifying CM

In the 5 mo data collection period, milk samples from 469 cows at VT and 203 cows at UKY were collected (Table 5.2). Clinical mastitis was identified on 49 occasions (26 cows) at VT and 36 occasions (24 cows) at UKY, with monthly incidence rates of 9.8 cases/mo at VT and 7.2 cases/mo at UKY. At VT, 35% of CM cases were caused by GP pathogens, 29% by GN pathogens, 14% each had no pathogen isolated or a mixed infection, 2% were classified as other infections, and 6% were inconclusive due to either contamination in culture or no sample being collected prior to treatment (Table 5.2). For UKY CM cases, 33% were caused by GP pathogens, 25% each had GN CM or no pathogen isolated, and the remaining 17% were mixed infections.

Records from 55,071 cow days across the 2 farms were used to assess model performance. The Se for detecting CM caused by any pathogen, or GN or GP pathogens ranged from 7% to 21%, and the Sp exceeded 99% for all models (Table 5.3). Using the ACM model, 18 CM cases were detected on the day of an alert (true positives), but 306 alerts did not relate to a CM case (false positives), resulting in a SR of 6%. On both farms, the Se was greater for the ACM model (21%), compared with the GN (Se = 9%) and GP models (Se = 7%). At UKY, the GN and GP model did not detect any cases of CM caused by the respective pathogen type, resulting in 0% Se for both models. The Sp was 100% due to the small number of alerts by these models in the 15,230 cow days observed. The FAR1000 was lowest for the GP model, with 0.1 and 0.9 false alerts per 1000 cow milkings for UKY and VT, respectively. The ACM model had a FAR1000

of 8.3 cows at UKY and 4.5 cows at VT. For the VT herd with 230 cows, 5 cows would need to be assessed over 4 milkings, whereas with the UKY herd size of 110 cows, the farm staff would have to assess up to 9 cows over 9 milkings based on the performance of these models.

Model performance for detecting and classifying IMI

Intramammary infection status was available for 324 cow days, through assessment of milk samples collected from alerted and randomly selected cows at VT and UKY. Of the randomly sampled cows, 7 cows each from VT and UKY had at least 1 quarter with a contaminated sample and could not be categorized into a pathogen group, so were removed from analyses. At VT, 27 (42%) randomly sampled cows had no pathogen isolated from all quarters, leaving 37 (58%) cows with a pathogen isolated in at least 1 quarter, most of which were GP (n = 30; Table 5.2). Twelve randomly sampled cows from UKY were culture-negative (36%), and 21 cows (64%) had an IMI, most of which were caused by GP pathogens (n = 18).

The ACM model generated more alerts than the GN and GP models on both farms. This was especially true for UKY, where 135 cow alerts were generated for the ACM model, and in 5 mo, just 5 and 2 cows alerted for the GN and GP models, respectively (Table 5.2). Bacteriological outcomes associated with each alert are presented in Figure 5.1. Up to 29% of alerts at VT and 33% at UKY had inconclusive bacteriology, due to contamination or failure to collect samples. Using IMI as the gold standard for evaluating model performance, the Se of detecting any IMI, or IMI caused by GN or GP pathogens for both farms combined was 69%, 55% and 21%, respectively (Table 5.4). Specificity was lower for the ACM model (28%), compared with the Gram-specific models (GN Sp = 52%, GP Sp = 60%).

For VT cows, 65 ACM alerts were for cows that had an IMI, and 37 randomly sampled cows that did not alert were IMI-positive cows, resulting in 64% Se for the ACM model. Of the IMI-negative cows, 72 had an ACM alert and 27 did not alert (Sp = 27%). The PPV was 47% and

NPV was 42% for the ACM model. The performance of the ACM model was slightly better for UKY cows, with Se of 75%, Sp of 31%, PPV of 70% and NPV of 37%. The Se of detecting GN or GP IMI at VT was 60% and 30% for GN and GP models, respectively, whereas both models had 0% Se at UKY. Specificity was lower at VT (Sp of GN model = 43% and GP model = 53%), than UKY (86 to 87%). Across both farms, the PPV was < 33% for the GN and GP models, whereas the NPV was > 93% for the GN model, and between 42 and 51% for the GP model.

DISCUSSION

Multiple regression models, derived from a dataset containing unmanipulated, real-farm data, were used to indicate ACM, or cases of CM caused by GN or GP pathogens, from cows on 2 research farms over a 5 mo period. The incidence of CM in both herds was high (9.8 and 7.2 cases/mo at VT and UKY, respectively), but some cases were recurring clinical episodes from the same quarter or multiple cases within a cow. Across the 2 herds, the proportion of pathogen types causing CM were similar, with approximately 35% GP, and 25% GN pathogens. The number of CM cases with no pathogen isolated was greater at UKY (25%) compared with VT (14%), some of which might reflect bacterial death during storage and shipping for UKY milk samples, whereas sample delivery for VT was more frequent and of shorter duration. In addition, sample contamination was an issue, with 21% of cows having inconclusive bacteriology at each farm. A study of this nature relies on milking staff to collect milk samples using aseptic technique, and though training was provided initially, limited experience, and staff turnover could have contributed to the high contamination rate. Cows with an inconclusive result were not available for model evaluation.

Model parameters

The models derived from the real-farm dataset in this study contained different, and fewer, parameters, compared with models previously derived (Chapter 3). Using a dataset that had 50% CM prevalence, conductivity, lactose and protein percent, and lying time slopes, were significant parameters remaining in the ACM model (Chapter 3). Changes in fat and lactose percent, and step activity, were indicators of GN CM, and parameters in the original models for GP CM included changes in conductivity, SCC, lactose and protein % and number of lying bouts (Chapter 3). Apart from change in fat percent in the ACM model, no other milk components were significant indicators of ACM, GN or GP. Surprisingly, lactose percent was not a significant indicator of CM in the current study. Lactose concentration in milk is sensitive to the effects of mastitis, and was the second most important predictor of mastitis after SCC in a recent study (Ebrahimie et al., 2018). When below 4.8%, the risk of elevated SCC and IMI is greater (Nielsen et al., 2005). Using the slope approach, we expected the change in lactose percent in the 7 d prior to CM detection to be important for CM detection, especially GN CM because of the sudden, acute nature of these infection types (Schukken et al., 2011).

The only behavioral indicator in the current models was a change in step activity for indicating GN. Previously lying time, activity, and the number of lying bouts, were important in predicting any CM case or Gram-specific CM (Chapter 3). The absence of activity measures in GP models is corroborated by a study that demonstrated more pronounced changes in activity leading up to GN CM, than in cows with GP CM (Stangaferro et al., 2016). Without considering pathogen type, Yeiser (2011) reported that cows developing CM had greater lying time 5 d prior and reduced step activity 2 d prior to onset of CM, compared with clinically healthy animals. The importance of both activity and milk parameters as predictors of Gram-specific CM cases was less apparent in the current study, but this theory should not be disregarded. Because models were derived using a small number of CM cases (10 GN, 22 GP

and 59 total CM cases), it was unlikely that the variation in the different types of CM within the GP and GN classifications was well represented.

Conductivity and milk yield featured as indicators of all 3 models derived from the real-farm dataset. Since the 1960's, conductivity has been used in studies to indicate mastitis, and later, inline sensors measuring conductivity were incorporated in AMS for mastitis detection (Rutten et al., 2013). Electrical conductivity is a useful indicator of mastitis; firstly, due to the physiological impacts of mastitis (increased concentration of ions in milk within an infected gland), and secondly, the ease of measuring conductivity. However, conductivity can also be affected by factors other than mastitis, including temperature, milk fat content and the milk fraction assessed (Nielen et al., 1992). In AMS, conductivity is measured at the quarter level, providing opportunities to increase the Se of detecting CM by comparing quarter conductivity within a cow at the same milking, or by comparing measurements across successive milkings (Claycomb et al., 2009; Hogeveen et al., 2010). Though we only had cow-based measures, our time series approach aimed to take advantage of the change in conductivity over time as an infection establishes and the inflammatory response develops. Milk yield declines due to mastitis, but the magnitude depends on severity of infection and the type of pathogen causing infection, as well as parity and stage of lactation of the cow (Gröhn et al., 2004). Milk yield serves as a non-specific indicator of mastitis, because milk yield depression can occur for many reasons (e.g., dietary changes, estrus, metabolic diseases, lameness, heat stress). Therefore, milk yield alone is not a good indicator of CM, but when used in combination with other parameters, such as conductivity, SCC, and milk color, some value has been demonstrated for mastitis detection (Kamphuis et al., 2008; Mollenhorst et al., 2010; Jensen et al., 2016).

Model performance for detecting and classifying CM

For detecting CM, model Se estimates were low (7 to 21%), and Sp were high (> 99%). High Sp often occurs at the expense of Se, or vice versa, because Se and Sp are inversely related.

Though some CM cases were alerted by 1 of the models on the day CM was observed (n = 22), the remaining 63 CM cases were not detected by any model. Missing data had a great impact on the number of cases that were not alerted by any model. Within the 55,071 cow day data collected over 5 mo, there were 426 false positive alerts, but still, because of the abundance of cow days that were not associated with an alert, Sp was perceived to be high. Success rate and FAR1000 are often preferred as indicators of model performance for detecting CM within a practical farm setting (Hogeveen et al., 2010). The SR ranged from 2.4% to 5.6%, meaning that at least 94% of alerts were not related to a true case of CM. Even though the FAR1000 for each model was low, corresponding to approximately 1 alert per milking, too many true cases of CM were missed by the models to make their use worthwhile.

Other authors have reported mastitis detection models with Se in the range of 55% to 89%, and Sp from 56% to 99%; however, few had high combined Se and Sp, and those that do were limited by sample size, or used an impractically wide time window (reviewed by Rutten et al., 2013). The goal of a CM detection model is to reliably detect a CM case before clinical signs of mastitis are observed, but not detect too early, otherwise a producer may perceive the alert as false and lose trust in the system (Hogeveen et al., 2010). Using this logic, we set the time window to 24 h for a positive alert to coincide with the CM event. Time windows of up to 17 d have been reported (de Mol et al., 1997), and as the time window increases, model performance usually increases, because there is a greater chance of an alert coinciding with the CM event (Hogeveen et al., 2010). Even with adjustments to the time window, our model's combined Se and Sp were unlikely to reach the levels of 80% and 99% respectively, necessary for effective mastitis detection systems in AMS (Hogeveen et al., 2010). Instead, adjustment of model parameter estimates or a more sophisticated modeling approach are likely to improve model performance.

When considering performance across both farms, the ACM model had better Se estimates (21%), compared with the Gram-specific models (7 to 9% for GP and GN models, respectively). This result must be interpreted with caution, as a farm effect was observed. Even when we adjusted the threshold for UKY, there were still only a combined total of 7 alerts between the 2 models. Possibly, further reduction in the threshold may have increased the number of alerts, but based on the success of the model at VT, many were likely to be unsuccessful. Failure of the GN and GP models to detect any CM case caused by the respective pathogen at UKY resulted in Se of 0%, skewing downward the Se estimate across the 2 farms. At VT, the 3 models were similar in Se, ranging from 12 to 18%. Though not the case, we expected that separate pathogen models would have superior performance in detecting Gram-specific CM, due to the differences in pathogenesis of GN and GP bacteria reflected in milk composition and cow behavior, as reported previously by our group (Chapter 3; Tholen, 2012) and elsewhere (Schukken et al., 2011; White et al., 1986; Milne et al., 2003). Accuracy estimates of 55% to 73% have been reported in other studies using sensor data to differentiate CM cases by Gram-status (Steeneveld et al., 2009; Kamphuis et al., 2011). We did not present accuracy, as it would have been biased by the Sp, or the successful classification of non-cases. More importantly, our approach is not directly comparable, as our Gram-specific models aimed to detect CM and indicate pathogen type in the same model (i.e., CM status was unknown), whereas Steeneveld and colleagues (2009) took a known case of CM and generated a probability of being caused by a GP or GN pathogen.

Improvements in model performance may be made by using more CM cases for model training and testing (Kamphuis et al., 2011), and subsequently, further classification of GP cases into *Staph. aureus*, streptococci and minor pathogens including CNS. Infections caused by different species within the GP classification vary in terms of pathogenicity and severity, and therefore, effects on milk parameters, especially, are expected to differ. Steeneveld et al., (2009)

classified CM cases caused by *Staph. aureus*, environmental streptococci and *E. coli* with 53% accuracy, but with less CM cases available (n = 140), the correct classification rate was 28% (Kamphuis et al., 2011). Both studies attributed the relatively poor performance of the classification models to the limited availability of CM cases due to these specific pathogens.

Model performance for detecting and classifying IMI

Although our models were designed primarily to detect CM cases, a secondary objective of the study was to evaluate model performance for detecting IMI. Our evaluation demonstrated that no model had high combined Se and Sp for detecting IMI. Given the model performance for detecting CM, this result was not surprising. For both farms, almost equal proportions of cows with an ACM alert were IMI-negative and IMI-positive (VT PPV = 47%, UKY PPV = 56%). Furthermore, NPV, or the proportion of cows that were confirmed IMI-negative without an alert (randomly sampled cows), was less than 50% for all but the GN model. Therefore, approximately half of the alerted cows had an IMI, as did the randomly sampled cows, implying that the models could have been predicting IMI purely by chance.

It must be acknowledged that differences exist in the definitions of CM, subclinical mastitis and IMI. Clinical mastitis is the visible presentation of abnormal milk, implying inflammatory processes within the gland are occurring in response to bacterial infection, and subclinical mastitis infers inflammation without visible changes to the milk or mammary gland. Neither CM or subclinical mastitis definitions require the presence of an etiological agent. Conversely, IMI infers the presence of bacteria in the milk, and does not stipulate whether inflammation is involved (Berry and Meaney, 2006). However, because IMI (i.e., bacteria) can induce inflammation and establish a subclinical infection, our models for detecting various types of CM might have provided value in categorization of IMI types, if the time series changes in milk composition that occur because of inflammation were well represented by the models.

Though the current models had limited effect in predicting IMI, improvements in our models for CM detection may pave the way for better IMI or subclinical mastitis detection.

CONCLUSIONS

Overall, the Se of detecting CM by each of the models was poor (7% to 21%), and the SR of an alert was less than 7%. The total number of alerts generated for more than 55,000 cow days was relatively small, contributing to the observed high Sp and low FAR1000. However, the proportion of successful alerts was unsatisfactory for these models to be useful in practice. Only 25% and 20% of CM cases coincided with an ACM alert at UKY and VT, respectively. When considering model application at VT, little difference was apparent in performance between the ACM model, and the Gram-specific, GN and GP models. However, no correct GN and GP alerts occurred at UKY, yielding 0% Se and skewing the Gram-specific model performance across the 2 farms. Even with a reduced cutoff threshold, GN and GP models developed using the VT herd data were not adequate for CM detection in cows at UKY. Models were also unable to detect any IMI or Gram-specific IMI with high Se and Sp. The level of infection in each herd was similar between alerted cows and randomly sampled cows, implying that any IMI detected could have been by chance. Based on current performance, our models would provide little value as a mastitis detection system in a real-farm setting. A small number of cases were used to derive the Gram-specific models (10 to 22 CM cases), and fewer milk component and activity measures were incorporated in all models than our group has previously reported. Model parameters and their estimates in each model require modification, using larger datasets and further categorization especially for GP pathogens, to represent the variation in individual CM cases within a Gram-classification. Future studies should utilize more CM cases of each pathogen type, which may lead to improvements in detection performance, and the ability to distinguish between GN and GP CM on-farm in real-time.

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Table 5.1. The final models for all clinical mastitis cases (**ACM**), Gram-negative clinical mastitis cases (**GN**) and Gram-positive clinical mastitis cases (**GP**) and the number (n) of cases used to derive each model with the sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff.

Response	**Model**	**Slope estimate (± SE)**	***P* value**	**n**	**Cutoff**	**Se (%)**	**Sp (%)**
ACM				59	0.01	66.1	68.8
	Intercept	-8.16 ± 0.25	< 0.01				
	Parity	0.32 ± 0.09	< 0.01				
	Breed	-0.87 ± 0.36	0.01				
	Conductivity; d -7 to -1	0.87 ± 0.33	0.01				
	Milk fat; d -7 to -1	2.55 ± 1.02	0.01				
	Milk yield; d -7 to -1	-0.57 ± 0.10	< 0.01				
	Conductivity; d -7 to -4	2.35 ± 0.67	< 0.01				
	Conductivity; d -7 to -5	-0.96 ± 0.39	0.01				
	Milk yield; d -7 to -5	-0.15 ± 0.05	< 0.01				
GN				10	0.02	60.0	98.1
	Intercept	-10.95 ± 0.73	< 0.01				
	Parity	0.51 ± 0.23	0.03				
	Daily steps; d -7 to -2	-0.04 ± 0.01	< 0.01				
	Conductivity; d -7 to -3	3.54 ± 0.74	< 0.01				
	Milk yield; d -7 to -3	-0.56 ± 0.10	< 0.01				
GP				22	0.05	4.5	100.0
	Intercept	-8.41 ± 0.19	< 0.01				
	Conductivity; d -7 to -3	-1.82 ± 0.81	0.03				
	Milk yield; d -7 to -3	-0.42 ± 0.13	< 0.01				

Table 5.2. Summary of bacteriological outcomes including pathogen type classifications and bacterial groups or species isolated from: cows with naturally occurring clinical mastitis (**CM cases**); cows that were alerted by the all clinical mastitis cases model (**ACM alert**), Gram-negative pathogen model (**GN alert**) or Gram-positive pathogen model (**GP alert**); and cows selected for sampling at random (**RAND**) at the Virginia Tech Dairy (**VT**) and the University of Kentucky Dairy (**UKY**) in the 5 mo data collection period.

Bacteriological outcome	**Species**	**VT (n of cows)**					**UKY (n of cows)**				
		CM cases	**ACM alert**	**GN alert**	**GP alert**	**RAND**	**CM cases**	**ACM alert**	**GN alert**	**GP alert**	**RAND**
Gram-positive	CNS	5	35	20	7	18	5	46	3		14
	Strep. spp.	6	6	7	5	2	1	1	1		
	Staph. aureus	1		2	1	4	3	1			1
	Mixed GP	5	5	3		6	3	4			3
	Total	17	46	32	13	30	12	52	4	0	18
Gram-negative	*E. coli*	2	3			3	5	2			
	Klebsiella spp.			1							
	Serratia spp.	5	1				1	2			1
	Citrobacter spp.	1					1				
	Enterobacter		1				1	1			
	Unidentified GN	6	4	5	2	1	1	1			
	Total	14	9	6	2	4	9	6	0	0	1
Other	*Bacillus* spp.	1									
	Yeast		1								
	Total	1	1	0	0	0	0	0	0	0	0
No Pathogen Isolated		7	72	41	23	27	9	27	1	1	12
Contaminated		1	51	24	16	7		31			7
Mixed infection[1]		7	9	4	2	3	6	5			2
No samples collected		2	1					14		1	
Total		49	189	107	56	71	36	135	5	2	40

[1]Mixed infection includes cows with Gram-negative and Gram-positive pathogens isolated from either the same quarter or from different quarters within the same cow.

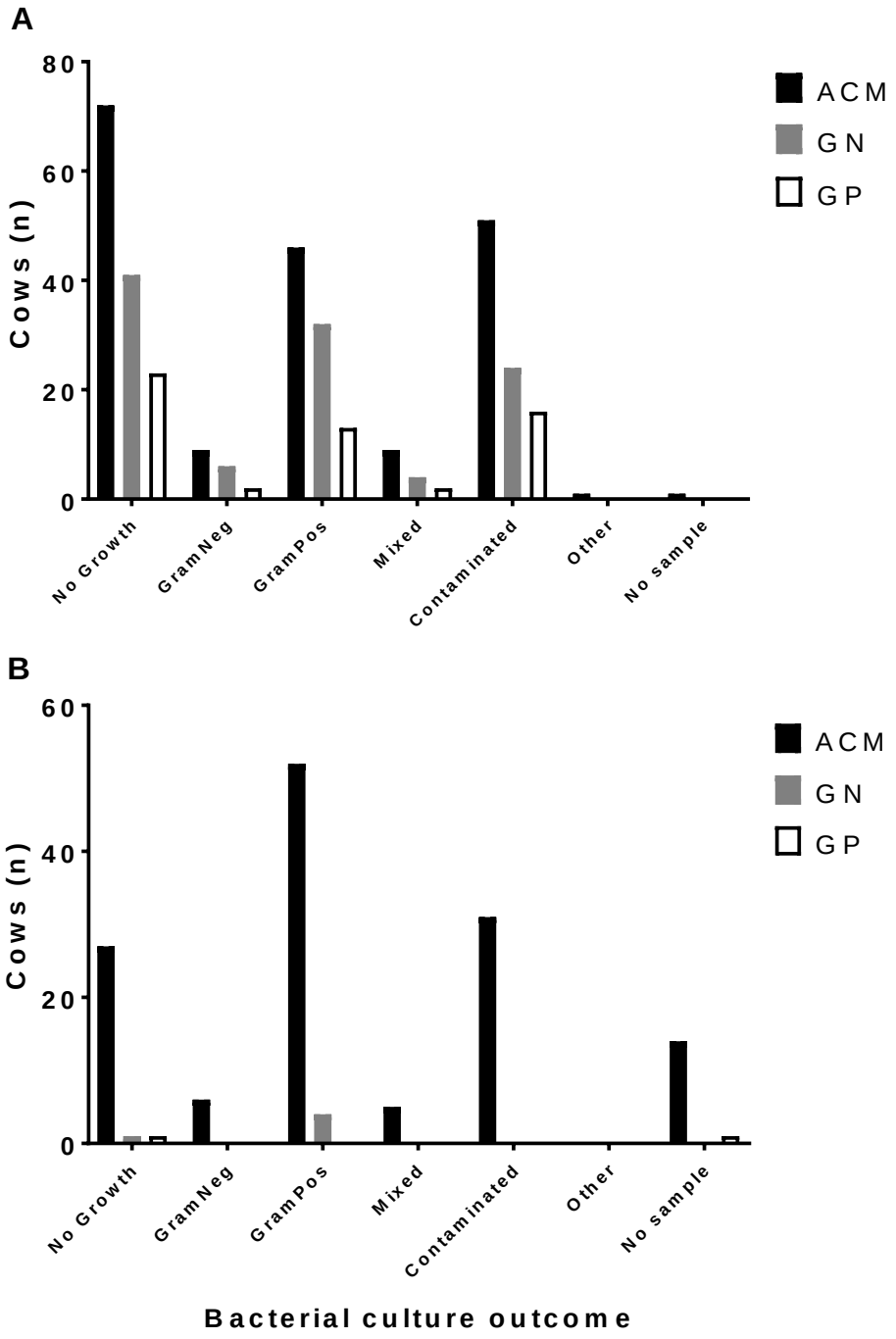
Table 5.3. Performance measures including sensitivity (**Se**), specificity (**Sp**), success rate (**SR**), and false alert rate per 1000 cow milkings (**FAR1000**) for clinical mastitis (**CM**) detection by the 3 models (**ACM** = all CM cases, **GN** = Gram-negative CM cases only, and **GP** = Gram-positive CM cases only), implemented on 2 farms (Virginia Tech Dairy (**VT**) and University of Kentucky Dairy (**UKY**)) for 5 mo.

Response	CM +		CM –		Se (%)	Sp (%)	SR (%)	FAR1000
	Alert	No alert	Alert	No alert				
VT								
ACM	9	40	180	39,612	18.4	99.5	4.8	4.5
GN	2	12	77	39,750	14.3	99.8	2.5	1.9
GP	2	17	36	39,788	11.8	99.9	5.3	0.9
UKY								
ACM	9	27	126	15,068	25.0	99.2	6.7	8.3
GN	0	9	5	15,216	0	100.0	0	0.3
GP	0	12	2	15,216	0	100.0	0	0.1
VT + UKY								
ACM	18	67	306	54,680	21.2	99.4	5.6	5.6
GN	2	21	82	54,966	8.7	99.9	2.4	1.5
GP	2	29	38	55,004	6.5	99.9	5.0	0.7

Table 5.4. Performance measures including sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**) and negative predictive value (**NPV**) for intramammary infection (**IMI**) detection by the 3 models (**ACM** = all CM cases, **GN** = Gram-negative CM cases only, and **GP** = Gram-positive CM cases only), implemented on 2 farms (Virginia Tech Dairy (**VT**) and University of Kentucky Dairy (**UKY**)) for 5 mo.

Response	**IMI +**		**IMI –**		**Se (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
	Alert	**No alert**	**Alert**	**No alert**				
VT								
ACM	65	37	72	27	63.7	27.3	47.4	42.2
GN	6	4	77	57	60.0	42.5	7.2	93.4
GP	13	30	27	31	30.2	53.4	32.5	50.8
UKY								
ACM	63	21	27	12	75.0	30.8	70.0	36.4
GN	0	1	5	30	0	85.7	0	96.8
GP	0	18	2	13	0	86.7	0	41.9
VT+UKY								
ACM	128	58	99	39	68.8	28.3	56.4	40.2
GN	6	5	82	87	54.5	51.5	6.8	94.6
GP	13	48	29	44	21.3	60.3	31.0	47.8

Figure 5.1. Number of cow alerts for the 3 mastitis detection models (**ACM** = all clinical mastitis cases, **GN** = Gram-negative clinical mastitis cases only, **GP** = Gram-positive clinical mastitis cases only) and their associated bacterial culture outcomes for cows at **A)** Virginia Tech Dairy Center and **B)** University of Kentucky Dairy.



CHAPTER 6. General conclusions and future research directions

Mastitis in dairy cows is a common and costly disease that adversely affects animal welfare and productivity, as well as milk quality. Mastitis prevention and detection are critical elements of mastitis control. On-farm strategies to prevent or reduce the severity of mastitis, and assist in the rapid detection of mastitis are warranted, to improve animal health outcomes, and for more judicious use of antimicrobial products on dairy farms. This dissertation outlined experimental studies in two areas of mastitis control: prevention (vaccination against coliform mastitis), and detection (mastitis detection and pathogen type indication using sensor data). As such, the general conclusions will be split into appropriate sections.

Vaccination against coliform mastitis

We hypothesized that vaccinated cows would exhibit quicker clinical recovery and fewer changes in lying behavior following induced *Escherichia coli* clinical mastitis (**CM**), compared with control (**CTL**) cows, and that the two J5 vaccines would elicit a similar antibody response. However, these hypotheses were not supported. Milk appearance, somatic cell score (**SCS**), bacterial counts, and the reduction in milk and component yields were not different between treatments and CTL at any post-challenge time point. The only clinical or behavioral differences observed were an earlier (by 3 h) peak vaginal temperature at 12 h post-challenge and less restless behavior at d 2 post-challenge in vaccinates, compared with CTL cows. Nevertheless, these minor differences between vaccinated and CTL animals did not correspond to any differences in clinical outcomes following challenge.

Secondly, cows vaccinated with Vaccine 1 (**V1**) had consistently greater serum antibodies, specifically IgG1 and IgG2, than cows vaccinated with Vaccine 2 (**V2**) and the unvaccinated CTL cows, showing a clear disparity between the 2 vaccines. The difference in antibody concentrations may have been related to variations in vaccine composition, such as the adjuvant, which was not disclosed by manufacturers. The J5-specific antibody titers are known

to decline as lactation progresses, so it was surprising to observe the divergent antibody response. However, the increased serum concentrations of IgG1 and IgG2 in V1 cows did not correspond to an improvement in clinical outcomes in these cows over the V2 or unvaccinated CTL groups. Additionally, a strengthened antibody response in multiparous cows, relative to primiparous heifers, was not associated with reduced severity of CM. Instead, the opposite effect was observed; primiparous heifers with a weaker antibody response, recovered faster than multiparous cows. These findings corroborate the theory that antibodies are not the sole driver of the enhanced immune response due to J5 vaccination. Alternatively, a growing body of evidence points to neutrophil recruitment and function having a critical role in defense against *E. coli*, in synergy with adaptive immunity (Hill, 1981; van Werven et al., 1997; Herry et al., 2017).

Our hypotheses were developed on considerable research on induced or observed CM in early lactation. In naturally occurring CM studies, vaccination reduced the incidence of CM in the first 90 d of lactation (Gonzalez et al., 1989; Cullor, 1991; Hogan et al., 1992). Following experimental mastitis, a protective effect in the first 30 DIM was demonstrated for some (Hogan et al., 1995, 2005; Tomita et al., 1998), but not all studies (Smith et al., 1999; Tomita et al., 2000). Our study differed in the timing of intramammary challenge, which occurred an average of 84 d after the last vaccine was administered to vaccinated cows at 14 DIM. Our results suggest that the effects of vaccination were diminished by 100 DIM. In early lactation, J5 vaccination is proposed to reverse the immunosuppressive state associated with a T-helper-2 (**Th-2**) response that promotes the production of a less effective opsonin, IgG1. Consequently, the vaccination reverts the immune response in early lactation to a pro-inflammatory state, driven by T-helper-1 (**Th-1**) cells, producing increased levels of IgG2, which plays a critical role in stimulating phagocytosis by neutrophils. Whereas, later in lactation, cows are already employing a Th-1 response and are biologically more resistant to

mastitis (Shafer-Weaver et al., 1999). Perhaps this explains the absence of clinical differences between vaccinated and unvaccinated cows in our study. To further support this notion, primiparous heifers had a faster rise and subsequent regression in SCC, and reduced peak bacterial counts, compared with multiparous cows, alluding to improved neutrophil recruitment and efficiency.

Future studies should investigate whether additional vaccine doses would provide any benefit for coliform CM occurring later in lactation, and focus on the effect of vaccination on neutrophil recruitment, function and synergy with the adaptive immune system. Hyperimmunization (6 vaccinations) has been proposed as an approach to increase J5-specific antibody titers beyond peak lactation, which reduced the incidence of naturally occurring severe CM, compared with cows that received 3 doses (Erskine et al., 2007). In addition to the immunization schedules for the 2 vaccines used in our study, the effects of an extra 3 vaccine doses could be explored. The schedule would recommend a vaccine dose at approximately d -60, -21 and d 14, as before, with the 4^{th} dose at 42 DIM, 5^{th} dose at 70 DIM and the 6^{th} dose at 98 DIM. If enough cows are available, the 3 dose regimen and 6 dose regimen for both vaccines could be tested against unvaccinated controls. All cows should be multiparous, to remove the effect of parity that was evident in our study, and preferably the same breed.

To investigate the activity of neutrophils throughout the acute stages of infection, an intramammary challenge study is necessary. Cows should be challenged after 120 DIM, so that sufficient time has passed since the last vaccination. Along with the clinical, behavioral, antibody and cytokine variables evaluated in the current study, the abundance and function of neutrophils should be investigated in a subset of the animals; specifically, differential cell counts using flow cytometry, and gene expression or *in vitro* assays to investigate neutrophil phagocytic ability and oxidative burst activity. This work would attempt to elucidate the

mechanisms of the J5 vaccine and particularly the theory that neutrophils play an important role in mediating the effects of vaccination, in combination with humoral immunity.

Ultimately, vaccine development for coliform vaccines should focus on prolonging the effects on neutrophil activity and J5-specific IgG2 to provide adequate protection in fewer doses. Additional doses will only be cost effective for farms that suffer from severe CM due to coliform bacteria throughout lactation. Vaccination is a management practice used on farms to supplement mastitis prevention, but farms with abundant CM cases due to coliform bacteria should first address hygiene issues.

Prevention of mastitis is best, but because cows live in an environment surrounded by bacteria, mastitis will continue to be a problem. Therefore, rapid, automated detection methods are necessary to identify cows with mastitis, and further, for judicious antimicrobial use, diagnosis of the etiological agent.

Mastitis detection and pathogen type indication using sensor data

The overall goal of this research was to develop Gram-specific models using time series changes in relevant milk and activity parameters, and test their ability to indicate CM caused by Gram-negative (**GN**) and Gram-positive (**GP**) pathogens. Gram-specific differentiation of CM cases has been emphasized as one of the leading priorities for mastitis research, especially in the current climate that advocates prudent antimicrobial use.

In the first study (Chapter 3), a case-control dataset was used to investigate univariate relationships between milk and activity sensor variables and all CM cases (**ACM**), or the categorized pathogen types (GN, GP, and no pathogen isolated (**NPI**)). We demonstrated that individually, some sensor variables were important for indicating the categorized CM cases, whereas the variation within ACM (and their etiological agent and pathogenesis) likely contributed to the lack of significant indicators of CM. From this work, we gained knowledge

about separate pathogen types and their relationships with milk and activity parameters over various day ranges in the 7 d prior to CM detection, to then apply in the development of multiple regression models.

As the main objective of Chapter 3, we described the development of multiple regression models for the CM responses (ACM, GN, GP, and NPI) at 3 different baselines. We evaluated these models initially using the training dataset (internal evaluation), and, subsequently, in a test dataset representing real-farm, unmanipulated data in Chapter 4.

Using the model training dataset, internal evaluation demonstrated a benefit of categorizing CM by GN, GP and NPI cases, as the accuracy exceeded 80% for all GP models, and for at least 1 GN and NPI model, whereas 75% accuracy was reached by the best ACM model. However, performance was reduced in all models following independent evaluation, and the slight benefit of Gram-specific models over ACM was diminished, at least for GP and NPI cases. Only the GN model had superior performance (sensitivity (**Se**) and specificity (**Sp**) = 62%), over the ACM model (Se = 56%, Sp = 52%), but none of our models had the required level of performance in mastitis detection systems for automated milking systems (Se = 80%, Sp = 99%; Hogeveen et al., 2010). Nevertheless, it is worth pointing out that no model reported in the literature meets these targets.

Gram-negative models were consistently identified to have the greatest detection accuracy using d -3 as the baseline for time series data incorporated into the model, which concurs with the sudden, acute nature of GN infections. Gram-positive models appeared to benefit from a baseline between 7 to 10 d prior to CM detection, again in line with expectations of an infection that progresses more gradually (than GN infections), and may be in a subclinical phase prior to CM detection. Not surprisingly, NPI cases were inconsistent in terms of model performance across datasets and the best baseline for detection. These infection types can occur for many

reasons, which vary between herds. Therefore, a detection model for these NPI cases is unlikely to be successful, but faster detection via other approaches (e.g., on-farm culture systems) should become a priority to restrict antimicrobial use to CM cases that are more likely to benefit from treatment.

Following independent evaluation, the GP model Se was < 32% for all models, a reduction in Se estimated in the internal evaluation (82%). A potential issue of our pathogen categorization is that we included the minor GP pathogen, coagulase-negative staphylococci (CNS), in with major pathogens, *Staph. aureus* and environmental streptococci. Infections due to major and minor pathogens differ in terms of pathogenesis, and are likely to be reflected differently in milk and activity parameters. Inclusion of minor pathogens within the GP classification was not conducive to model derivation, and might explain the poor repeatability of the GP model. Pathogen-specific models are suggested to improve detection performance, but current estimates of accuracy in classifying *Staphylococcus aureus*, environmental streptococci and *E. coli*, are limited (28% to 52%; Steeneveld et al., 2009; Kamphuis et al., 2011), which is most likely due to the difficulty in accessing sufficient numbers of CM cases to develop and test models.

An insufficient number of CM cases is a common challenge identified by researchers working in the field of mastitis detection using sensor technologies. The availability of CM cases with associated bacteriological information is limited, due to the difficulty in predicting when CM will occur, and the logistics of identifying the causative pathogen. Using an abundance of CM cases in large datasets is important to represent the variation in individual CM cases within a Gram-classification.

A secondary reason for the insufficient numbers of CM cases in total, and for each of the pathogen types investigated, is the number of missing observations in sensor data. In our study,

many CM cases were unavailable for use in deriving models, because of incomplete data. Initially, the case-control dataset had 268 CM cases, which declined to 170 cases with complete observations for the day ranges used. In turn, this provided only 50 CM cases for the ACM model derivation, and even less for the pathogen-specific models. We did not implement any data manipulation to make up for missing data, but future studies will need to consider this issue.

Differences in infection distribution between the datasets used for internal and external evaluation may also account for the poorer performance of models during the independent external evaluation. The model training dataset was built from a case-control study (Tholen, 2012), so approximately 50% of the records related to a CM case, and the other 50% were associated with controls (considered healthy non-cases). Conversely, the test dataset contained data for every cow (average 230 cows) and every day (690 d), without removing non-case data to balance cases and controls. As a result, the test dataset diluted out the CM cases, but reflected the typical level of CM seen in practice, which is much less than the 20 to 50% CM prevalence (by design) in the model training dataset. The differences between the 2 datasets highlighted a challenge with using case-control data for model derivation if those models are intended for on-farm application.

This leads us to Chapter 5, where we used the same modeling approach to derive new multiple regression models using the test dataset from Chapter 4, which consisted of real-farm data with a similar infection prevalence and distribution to the intended application. We hypothesized that using more suitable data to derive models would improve detection performance when these models were implemented on 2 farms (the model training herd, **VT;** and an independent herd, **UKY**). The new models contained fewer parameters than before, with electrical conductivity and milk yield featuring in all models, and milk composition and cow behavior variables, less important. After 5 mo of testing, the models proved to be unsuccessful in terms

of correct classification of CM cases (Se), and producing correct alerts (success rate [**SR**]). The maximum Se was 21% for the ACM model, meaning that approximately 1 in 5 CM cases were identified by an alert. The SR was 5%, so 95% of ACM alerts did not relate to a CM case. The false alert rate per 1000 milkings was 4.5 cows, which would be acceptable for a farmer, but only if a significant proportion of CM cases were being detected. Based on current performance, our models would provide little value as a mastitis detection system in a commercial farm setting.

Again, we faced similar challenges as in Chapter 3 and 4, which echo the difficulties reported in the literature. An insufficient number of CM cases were available to derive the Gram-specific models (10 to 22 CM cases), which contributes to their poor performance in detecting CM. The repeatability between independent herds was an issue for the Gram-specific models in our study, because even after the thresholds for UKY were reduced by a magnitude of 10, only only 7 cows alerted by the GN and GP models at UKY in the 5 mo study period. Clearly, the models required training and adjustments to be repeatable across farms.

Accuracy estimates of 55% to 73% have been reported in other studies using sensor data to differentiate CM cases by Gram-status (Steeneveld et al., 2009; Kamphuis et al., 2011). In the study by Steeneveld et al. (2009), 1,202 CM cases were used to validate models. Classification accuracy increased to 95% when a probability threshold of >0.90 was used to classify pathogens as positive. Our approach is not directly comparable because the Gram-specific models aimed to detect CM and indicate pathogen type in the same model (i.e., CM status was unknown), whereas Steeneveld and colleagues (2009) took a known case of CM and generated a probability of being caused by a GP or GN pathogen. Without access to large datasets for training models for Gram-specific CM detection, the best progress will be made by using current mastitis detection systems that generate an alert list, and use a ranking system to reduce the number of false positives (Steeneveld et al., 2010), or by using sequential models, that first

aim to detect CM, or use a known case of CM, to subsequently classify that pathogen as GN or GP (Steeneveld et al., 2009).

Continuation of this research should consider more sophisticated modeling approaches. One relatively recent study used a naïve Bayesian classifier, in combination with a dynamic linear model (Jensen et al., 2016), which detected CM with a Se of 80%, and a Sp of 81%. The dynamic linear model provides one step ahead forecasting, in line with the long term trend of the data, and the naïve Bayesian classifier better handles missing data and combines sensor data from a range of sources. This study has produced one of the highest combinations of Se and Sp reported in the literature, using a relatively large dataset and an acceptable time window range. Still, when sensitivity and specificity are < 90%, there lies a lot of room for false positives and false negatives on a daily basis when considering the whole herd and twice daily milking. For mastitis detection systems, the level of accuracy depends on what is most important to the farmer.

In the future, application of more complex machine learning techniques to the field of mastitis detection may be better able to draw trends out of the data, and handle missing observations, but the biological meaning can get lost within the model. This research extends far beyond the scope of this dissertation. Research into mastitis detection and diagnostic tools has been a constant focus for decades; the challenges around detecting a multifactorial, multi-phase disease such as mastitis suggests that it will continue for decades to come.

Conclusion

In conclusion, the work presented in this dissertation has encompassed two aspects of mastitis control in modern day dairy farms. By inducing *E. coli* mastitis in vaccinated and unvaccinated mid-lactating dairy animals, we have confirmed that the effect of J5 vaccination was diminished by mid-lactation, potentially providing support for additional vaccine doses in

enhancing mammary gland immunity on farms that have an abundance of severe cases of CM due to coliform bacteria. Additionally, our research supports previous hypotheses that antibodies are not the sole driver of immune responses to vaccination. Future vaccine efficacy studies should measure components related to cell-mediated, as well as humoral immunity, for a better understanding of the collaboration between different branches of the adaptive immune system following vaccination.

Secondly, we have added knowledge to the field of mastitis detection using sensor technologies. We suggest that categorizing CM cases by Gram-status has potential for improving CM detection, especially when considering slopes changes over an appropriate time range for the specific pathogen type. But, we highlight the challenges of: 1) access to datasets with sufficient CM cases to produce reliable models, 2) the abundance of minor pathogens, which should be excluded from the GP classification, and 3) the development of models that will be repeatable and provide value on independent farms. Regarding the first 2 points, we emphasize the need for pathogen-specific models, as Gram-specific models are insufficient, especially for dealing with few cases that do not represent the variation in different types of CM, within a classification. Lastly, more sophisticated models with the ability to continually forecast should improve predictions, better handle missing data and have greater applicability across farms. Milk and activity sensor data may be useful to enhance mastitis detection, in combination with other well-documented mastitis-related parameters. As of yet, on-farm sensor data are not accurate enough to inform mastitis management decisions.

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CHAPTER 7. APPENDICES

Supplementary Table 7.1 Univariate models for all clinical mastitis cases grouped together regardless of causative pathogen type for the 10 explanatory variables. For each model, the number of cases, intercept and slope estimates (± SE), corrected Akaike's Information Criterion (**AICc**), and the percentage of cases and controls identified correctly (accuracy), sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff are presented.

Explanatory Variable	Slope Range	N. cases	Intercept	Slope Estimate	AICc	*P* value	Cutoff	Accuracy (%)	Se (%)	Sp (%)
Fat %	d -7 to -1	157	0.02 ± 0.11	-0.22 ± 1.12	436	0.84	0.53	50.3%	1.9%	100.0%
	d -7 to -2	154	0.04 ± 0.12	-0.42 ± 0.98	423	0.67	0.51	52.8%	57.8%	47.6%
	d -7 to -3	147	0.01 ± 0.12	-1.12 ± 0.70	407	0.11	0.50	55.3%	62.6%	47.9%
	d -7 to -4	143	0.03 ± 0.12	-0.64 ± 0.53	395	0.23	0.51	54.6%	47.6%	61.9%
	d -7 to -5	129	0.00 ± 0.13	-0.49 ± 0.47	361	0.30	0.54	53.3%	10.1%	96.9%
Lactose %	d -7 to -1	157	0.03 ± 0.12	1.34 ± 2.92	436	0.65	0.51	52.9%	36.3%	69.9%
	d -7 to -2	154	0.05 ± 0.12	-0.44 ± 2.94	423	0.88	0.52	49.5%	1.9%	99.3%
	d -7 to -3	147	0.02 ± 0.12	0.49 ± 2.70	409	0.86	0.51	50.9%	15.6%	86.8%
	d -7 to -4	143	0.03 ± 0.12	1.10 ± 2.08	397	0.60	0.51	53.5%	44.1%	63.3%
	d -7 to -5	129	0.01 ± 0.13	-1.32 ± 1.79	362	0.46	0.52	54.5%	20.9%	88.3%
Protein %	d -7 to -1	157	0.03 ± 0.11	-3.27 ± 2.97	435	0.27	0.51	52.9%	49.0%	56.9%
	d -7 to -2	154	0.05 ± 0.12	-2.16 ± 2.86	423	0.45	0.51	52.2%	58.4%	45.6%
	d -7 to -3	147	0.03 ± 0.12	-1.73 ± 2.35	409	0.46	0.50	51.9%	66.0%	37.5%
	d -7 to -4	143	0.03 ± 0.12	0.47 ± 1.72	397	0.79	0.52	50.7%	7.7%	95.0%
	d -7 to -5	129	0.02 ± 0.13	1.39 ± 1.35	361	0.30	0.46	52.5%	94.6%	10.2%
Conductivity	d -7 to -1	157	0.05 ± 0.12	-0.79 ± 0.59	434	0.18	0.52	55.8%	36.3%	75.8%
	d -7 to -2	154	0.04 ± 0.12	-0.26 ± 0.52	424	0.62	0.52	52.6%	19.5%	87.2%
	d -7 to -3	147	0.01 ± 0.12	-0.35 ± 0.47	410	0.46	0.51	53.4%	35.4%	71.7%
	d -7 to -4	143	0.02 ± 0.12	0.16 ± 0.39	398	0.69	0.54	50.9%	2.8%	100.0%
	d -7 to -5	129	0.01 ± 0.13	-0.35 ± 0.32	361	0.27	0.51	53.3%	39.5%	67.2%
Milk yield	d -7 to -1	157	0.06 ± 0.12	0.09 ± 0.08	435	0.27	0.46	53.5%	95.5%	10.5%
	d -7 to -2	154	0.05 ± 0.12	0.05 ± 0.08	424	0.53	0.47	52.6%	98.7%	4.7%
	d -7 to -3	147	0.02 ± 0.12	0.06 ± 0.07	410	0.36	0.51	52.1%	31.3%	73.1%
	d -7 to -4	143	0.02 ± 0.12	0.04 ± 0.06	398	0.48	0.52	51.9%	15.4%	89.3%

	d -7 to -5	129	0.01 ± 0.13	-0.03 ± 0.05	362	0.60	0.50	52.5%	62.0%	43.0%
LogSCC	d -7 to -1	157	0.02 ± 0.12	0.05 ± 0.33	436	0.88	0.51	51.0%	10.8%	92.2%
	d -7 to -2	153	0.04 ± 0.12	0.00 ± 0.32	422	0.99	0.01	51.0%	100.0%	0.0%
	d -7 to -3	147	0.03 ± 0.12	-0.16 ± 0.31	409	0.60	0.51	51.9%	36.7%	67.4%
	d -7 to -4	143	0.04 ± 0.12	-0.26 ± 0.28	396	0.34	0.56	51.1%	4.2%	99.3%
	d -7 to -5	129	0.02 ± 0.13	-0.05 ± 0.23	360	0.82	0.49	51.8%	98.4%	4.0%
Activity	d -7 to -1	157	0.01 ± 0.12	-0.03 ± 0.02	433	0.13	0.54	53.2%	12.7%	94.8%
	d -7 to -2	154	0.03 ± 0.12	-0.02 ± 0.01	423	0.18	0.55	53.3%	11.0%	97.3%
	d -7 to -3	147	0.01 ± 0.12	-0.01 ± 0.01	410	0.35	0.52	53.1%	15.0%	91.7%
	d -7 to -4	143	0.02 ± 0.12	0.00 ± 0.01	398	0.84	0.51	52.7%	9.8%	96.4%
	d -7 to -5	129	0.01 ± 0.13	0.00 ± 0.01	362	0.86	0.52	51.0%	3.1%	99.2%
Lying time	d -7 to -1	54	-0.15 ± 0.19	0.01 ± 0.01	162	0.17	0.49	61.2%	40.7%	79.0%
	d -7 to -2	54	-0.14 ± 0.19	0.01 ± 0.01	164	0.51	0.45	55.2%	81.5%	32.3%
	d -7 to -3	52	-0.18 ± 0.19	0.00 ± 0.01	161	0.77	0.47	55.3%	17.3%	87.1%
	d -7 to -4	52	-0.15 ± 0.19	0.00 ± 0.00	159	0.65	0.47	57.1%	36.5%	75.0%
	d -7 to -5	51	-0.15 ± 0.19	0.00 ± 0.00	156	0.90	0.46	50.0%	82.4%	22.0%
Lying bouts	d -7 to -1	54	-0.14 ± 0.19	-0.07 ± 0.24	164	0.76	0.44	49.1%	100.0%	4.8%
	d -7 to -2	54	-0.14 ± 0.19	0.05 ± 0.19	164	0.81	0.47	57.8%	38.9%	74.2%
	d -7 to -3	52	-0.18 ± 0.19	0.00 ± 0.14	161	0.98	0.46	55.3%	1.9%	100.0%
	d -7 to -4	52	-0.13 ± 0.19	0.10 ± 0.12	158	0.42	0.49	57.1%	26.9%	83.3%
	d -7 to -5	51	-0.15 ± 0.19	0.06 ± 0.09	156	0.48	0.50	60.0%	21.6%	93.2%
Lying bout duration	d -7 to -1	54	-0.16 ± 0.19	0.05 ± 0.06	164	0.43	0.49	58.6%	27.8%	85.5%
	d -7 to -2	54	-0.14 ± 0.19	0.01 ± 0.05	164	0.92	0.47	55.2%	16.7%	88.7%
	d -7 to -3	52	-0.20 ± 0.20	0.02 ± 0.04	161	0.70	0.45	52.6%	65.4%	41.9%
	d -7 to -4	52	-0.16 ± 0.20	0.01 ± 0.03	159	0.75	0.48	55.4%	15.4%	90.0%
	d -7 to -5	51	-0.15 ± 0.19	0.01 ± 0.02	156	0.52	0.44	51.8%	88.2%	20.3%

Supplementary Table 7.2. Univariate models for Gram-negative clinical mastitis cases for the 10 explanatory variables. For each model, the number of cases, intercept and slope estimates (± SE), corrected Akaike's Information Criterion (**AICc**), and the percentage of cases and controls identified correctly (accuracy), sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff are presented.

Explanatory Variable	Slope Range	N. cases	Intercept	Slope Estimate	AICc	*P* value	Cutoff	Accuracy (%)	Se (%)	Sp (%)
Fat %	d -7 to -1	37	-2.08 ± 0.51	1.32 ± 1.74	223	0.45	0.11	62.9%	73.0%	61.5%
	d -7 to -2	37	-2.05 ± 0.50	1.71 ± 1.50	221	0.25	0.09	60.8%	75.7%	58.7%
	d -7 to -3	35	-2.13 ± 0.57	0.82 ± 1.05	209	0.44	0.18	65.6%	74.3%	64.5%
	d -7 to -4	35	-2.11 ± 0.57	0.99 ± 0.79	206	0.21	0.07	58.2%	82.9%	54.7%
	d -7 to -5	33	-2.10 ± 0.57	0.94 ± 0.71	193	0.19	0.16	61.9%	75.8%	59.8%
Lactose %	d -7 to -1	37	-2.07 ± 0.53	5.37 ± 4.52	222	0.24	0.16	66.5%	70.3%	65.9%
	d -7 to -2	37	-2.08 ± 0.52	8.57 ± 4.39	219	0.05	0.16	68.4%	73.0%	67.8%
	d -7 to -3	35	-2.12 ± 0.58	4.99 ± 4.75	208	0.29	0.17	66.7%	74.3%	65.6%
	d -7 to -4	35	-2.09 ± 0.56	0.93 ± 3.52	207	0.79	0.19	63.8%	74.3%	62.3%
	d -7 to -5	33	-2.10 ± 0.52	-2.41 ± 2.86	194	0.40	0.12	59.5%	78.8%	56.7%
Protein %	d -7 to -1	37	-2.09 ± 0.52	-4.90 ± 5.20	223	0.35	0.15	62.6%	73.0%	61.2%
	d -7 to -2	37	-2.05 ± 0.49	-1.90 ± 4.65	222	0.68	0.17	61.8%	73.0%	60.2%
	d -7 to -3	35	-2.13 ± 0.57	-0.65 ± 4.06	209	0.87	0.07	61.2%	77.1%	59.0%
	d -7 to -4	35	-2.12 ± 0.56	2.35 ± 2.81	207	0.40	0.16	61.7%	77.1%	59.5%
	d -7 to -5	33	-2.09 ± 0.55	3.12 ± 2.10	192	0.14	0.13	60.3%	78.8%	57.6%
Conductivity	d -7 to -1	37	-2.07 ± 0.52	-0.55 ± 0.85	223	0.52	0.18	69.0%	67.6%	69.2%
	d -7 to -2	37	-2.07 ± 0.51	-1.03 ± 0.84	221	0.22	0.16	65.2%	73.0%	64.2%
	d -7 to -3	35	-2.18 ± 0.59	-1.39 ± 0.81	207	0.09	0.17	70.2%	74.3%	69.6%
	d -7 to -4	35	-2.15 ± 0.56	-1.48 ± 0.69	203	0.03	0.11	63.3%	82.9%	60.5%
	d -7 to -5	33	-2.11 ± 0.56	-0.80 ± 0.53	192	0.13	0.17	67.7%	75.8%	66.5%
Milk yield	d -7 to -1	37	-2.08 ± 0.51	0.00 ± 0.12	224	0.99	0.19	63.2%	73.0%	61.9%
	d -7 to -2	37	-2.06 ± 0.49	-0.01 ± 0.12	222	0.93	0.08	61.3%	73.0%	59.6%
	d -7 to -3	35	-2.14 ± 0.56	0.12 ± 0.09	208	0.20	0.08	61.3%	82.9%	58.4%
	d -7 to -4	35	-2.11 ± 0.56	0.08 ± 0.10	207	0.31	0.10	61.5%	80.0%	58.9%
	d -7 to -5	33	-2.09 ± 0.56	-0.04 ± 0.17	194	0.62	0.17	61.1%	78.8%	58.5%

LogSCC	d -7 to -1	37	-2.09 ± 0.52	0.12 ± 0.54	224	0.83	0.10	62.3%	73.0%	60.8%
	d -7 to -2	37	-2.05 ± 0.50	0.07 ± 0.53	222	0.89	0.19	64.3%	70.3%	63.5%
	d -7 to -3	35	-2.12 ± 0.57	-0.30 ± 0.55	209	0.59	0.16	62.2%	77.1%	60.2%
	d -7 to -4	35	-2.09 ± 0.57	-0.13 ± 0.44	207	0.78	0.18	62.1%	77.1%	59.9%
	d -7 to -5	33	-2.08 ± 0.57	0.11 ± 0.35	194	0.76	0.08	59.2%	78.8%	56.3%
Activity	d -7 to -1	37	-2.10 ± 0.52	-0.02 ± 0.02	223	0.39	0.15	63.2%	73.0%	61.9%
	d -7 to -2	37	-2.06 ± 0.49	0.00 ± 0.02	222	0.98	0.07	61.3%	73.0%	59.6%
	d -7 to -3	35	-2.15 ± 0.55	0.02 ± 0.02	207	0.13	0.15	63.4%	77.1%	61.5%
	d -7 to -4	35	-2.13 ± 0.55	0.02 ± 0.01	203	0.08	0.17	66.8%	77.1%	65.3%
	d -7 to -5	33	-2.17 ± 0.60	0.03 ± 0.01	187	0.01	0.12	62.3%	81.8%	59.4%
Lying time	d -7 to -1	23	-1.48 ± 0.25	0.02 ± 0.01	116	0.05	0.22	72.4%	52.2%	77.4%
	d -7 to -2	23	-1.43 ± 0.24	0.01 ± 0.01	118	0.24	0.23	73.3%	26.1%	84.9%
	d -7 to -3	23	-1.38 ± 0.23	0.00 ± 0.01	119	0.85	0.21	75.4%	17.4%	90.1%
	d -7 to -4	23	-1.39 ± 0.24	0.00 ± 0.00	117	0.31	0.20	57.1%	60.9%	56.2%
	d -7 to -5	22	-1.40 ± 0.24	0.00 ± 0.00	114	0.46	0.18	36.4%	95.5%	21.6%
Lying bouts	d -7 to -1	23	-1.39 ± 0.23	0.09 ± 0.30	120	0.76	0.20	56.9%	52.2%	58.1%
	d -7 to -2	23	-1.40 ± 0.23	0.09 ± 0.24	120	0.72	0.19	36.2%	82.6%	24.7%
	d -7 to -3	23	-1.38 ± 0.23	-0.04 ± 0.18	119	0.83	0.20	47.4%	65.2%	42.9%
	d -7 to -4	23	-1.37 ± 0.24	-0.08 ± 0.16	118	0.59	0.21	63.4%	43.5%	68.5%
	d -7 to -5	22	-1.40 ± 0.24	-0.13 ± 0.11	113	0.26	0.18	46.4%	81.8%	37.5%
Lying bout duration	d -7 to -1	23	-1.42 ± 0.24	0.04 ± 0.07	119	0.56	0.19	45.7%	69.6%	39.8%
	d -7 to -2	23	-1.40 ± 0.24	0.01 ± 0.06	120	0.91	0.20	62.9%	30.4%	71.0%
	d -7 to -3	23	-1.38 ± 0.24	0.01 ± 0.05	119	0.91	0.21	79.8%	8.7%	97.8%
	d -7 to -4	23	-1.37 ± 0.24	0.01 ± 0.04	118	0.79	0.20	39.3%	78.3%	29.2%
	d -7 to -5	22	-1.40 ± 0.24	0.03 ± 0.03	113	0.31	0.17	37.3%	95.5%	22.7%

Supplementary Table 7.3. Univariate models for Gram-positive clinical mastitis cases for the 10 explanatory variables. For each model, the number of cases, intercept and slope estimates (± SE), corrected Akaike's Information Criterion (**AICc**), and the percentage of cases and controls identified correctly (accuracy), sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff are presented.

Explanatory Variable	Slope Range	N. cases	Intercept	Slope Estimate	AICc	*P* value	Cutoff	Accuracy (%)	Se (%)	Sp (%)
Fat %	d -7 to -1	31	-2.21 ± 0.19	-0.95 ± 1.79	207	0.59	0.10	57.7%	54.8%	58.1%
	d -7 to -2	31	-2.18 ± 0.19	-1.10 ± 1.56	205	0.48	0.10	42.9%	64.5%	40.4%
	d -7 to -3	30	-2.17 ± 0.19	-0.51 ± 1.06	199	0.63	0.10	34.4%	80.0%	29.1%
	d -7 to -4	29	-2.17 ± 0.20	0.12 ± 0.83	193	0.88	0.11	89.4%	6.9%	98.8%
	d -7 to -5	28	-2.11 ± 0.20	0.50 ± 0.72	183	0.49	0.14	88.7%	7.1%	98.7%
Lactose %	d -7 to -1	31	-2.2 ± 0.19	-0.49 ± 4.84	208	0.92	0.01	10.0%	100.0%	0.0%
	d -7 to -2	31	-2.17 ± 0.19	-2.69 ± 4.96	205	0.59	0.11	77.7%	25.8%	83.7%
	d -7 to -3	30	-2.19 ± 0.20	-3.94 ± 4.19	198	0.35	0.11	72.9%	36.7%	77.0%
	d -7 to -4	29	-2.18 ± 0.20	-2.17 ± 3.39	193	0.52	0.12	87.9%	17.2%	96.0%
	d -7 to -5	28	-2.11 ± 0.20	-1.29 ± 2.87	183	0.65	0.12	85.2%	21.4%	93.0%
Protein %	d -7 to -1	31	-2.20 ± 0.19	1.06 ± 4.71	208	0.82	0.11	88.4%	3.2%	97.8%
	d -7 to -2	31	-2.17 ± 0.19	0.54 ± 4.68	206	0.91	0.10	15.6%	96.8%	6.3%
	d -7 to -3	30	-2.20 ± 0.20	4.27 ± 3.87	198	0.27	0.12	79.7%	23.3%	86.2%
	d -7 to -4	29	-2.17 ± 0.20	1.07 ± 2.80	193	0.70	0.10	37.2%	72.4%	33.2%
	d -7 to -5	28	-2.10 ± 0.20	1.23 ± 2.16	183	0.57	0.10	27.6%	89.3%	20.1%
Conductivity	d -7 to -1	31	-2.18 ± 0.19	-1.27 ± 0.91	206	0.16	0.11	75.2%	32.3%	79.9%
	d -7 to -2	31	-2.17 ± 0.19	-0.62 ± 0.89	205	0.49	0.11	76.8%	25.8%	82.7%
	d -7 to -3	30	-2.17 ± 0.19	-0.33 ± 0.78	199	0.67	0.11	81.8%	13.3%	89.7%
	d -7 to -4	29	-2.17 ± 0.20	-0.13 ± 0.64	193	0.84	0.09	11.0%	100.0%	0.8%
	d -7 to -5	28	-2.10 ± 0.20	-0.26 ± 0.52	183	0.62	0.11	59.9%	53.6%	60.7%
Milk yield	d -7 to -1	31	-2.16 ± 0.19	0.16 ± 0.13	206	0.24	0.11	74.8%	38.7%	78.9%
	d -7 to -2	31	-2.16 ± 0.19	0.06 ± 0.13	206	0.64	0.09	14.6%	96.8%	5.2%
	d -7 to -3	30	-2.17 ± 0.19	-0.05 ± 0.12	199	0.64	0.08	11.6%	100.0%	1.5%
	d -7 to -4	29	-2.17 ± 0.20	0.02 ± 0.09	193	0.85	0.11	88.3%	3.4%	98.0%
	d -7 to -5	28	-2.13 ± 0.21	0.12 ± 0.08	181	0.15	0.12	73.9%	39.3%	78.2%

LogSCC	d -7 to -1	31	-2.19 ± 0.19	-0.09 ± 0.57	208	0.88	0.10	47.4%	87.1%	43.0%
	d -7 to -2	31	-2.16 ± 0.19	-0.04 ± 0.53	205	0.95	0.01	10.3%	100.0%	0.0%
	d -7 to -3	30	-2.18 ± 0.20	0.23 ± 0.46	199	0.61	0.15	90.0%	3.3%	100.0%
	d -7 to -4	29	-2.17 ± 0.20	-0.34 ± 0.46	192	0.46	0.10	42.9%	69.0%	39.9%
	d -7 to -5	28	-2.10 ± 0.20	-0.29 ± 0.37	182	0.42	0.13	83.5%	14.3%	92.1%
Activity	d -7 to -1	31	-2.19 ± 0.19	0.01 ± 0.03	207	0.69	0.15	90.3%	3.2%	100.0%
	d -7 to -2	31	-2.17 ± 0.19	0.01 ± 0.02	206	0.79	0.12	89.7%	3.2%	99.6%
	d -7 to -3	30	-2.18 ± 0.20	-0.01 ± 0.02	199	0.45	0.12	88.0%	16.7%	96.2%
	d -7 to -4	29	-2.21 ± 0.20	-0.02 ± 0.01	191	0.13	0.11	78.8%	31.0%	84.3%
	d -7 to -5	28	-2.15 ±0.21	-0.01 ± 0.01	181	0.10	0.11	71.2%	42.9%	74.7%
Lying time	d -7 to -1	12	-2.17 ± 0.31	0.00 ± 0.01	81	0.79	0.10	33.6%	83.3%	27.9%
	d -7 to -2	12	-2.16 ± 0.31	0.00 ± 0.01	81	0.84	0.10	31.0%	83.3%	25.0%
	d -7 to -3	11	-2.24 ± 0.32	0.00 ± 0.01	76	0.76	0.10	65.8%	36.4%	68.9%
	d -7 to -4	11	-2.35 ± 0.36	0.01 ± 0.01	72	0.08	0.09	54.5%	81.8%	51.5%
	d -7 to -5	11	-2.42 ± 0.38	0.01 ± 0.01	70	0.03	0.10	64.5%	72.7%	63.6%
Lying bouts	d -7 to -1	12	-2.16 ± 0.31	0.22 ± 0.40	81	0.58	0.12	82.8%	33.3%	88.5%
	d -7 to -2	12	-2.16 ± 0.31	0.09 ± 0.31	81	0.77	0.10	35.3%	83.3%	29.8%
	d -7 to -3	11	-2.24 ± 0.32	0.13 ± 0.23	76	0.57	0.10	67.5%	54.5%	68.9%
	d -7 to -4	11	-2.24 ± 0.33	0.23 ± 0.18	74	0.20	0.09	47.3%	81.8%	43.6%
	d -7 to -5	11	-2.27 ± 0.34	0.17 ± 0.14	74	0.20	0.1.0	57.3%	63.6%	56.6%
Lying bout duration	d -7 to -1	12	-2.18 ± 0.31	0.04 ± 0.09	81	0.67	0.11	74.1%	41.7%	77.9%
	d -7 to -2	12	-2.22 ± 0.32	0.05 ± 0.07	81	0.48	0.12	82.8%	33.3%	88.5%
	d -7 to -3	11	-2.24 ± 0.33	0.01 ± 0.07	76	0.94	0.10	86.8%	9.1%	95.1%
	d -7 to -4	11	-2.28 ± 0.34	0.03 ± 0.05	76	0.56	0.12	83.0%	18.2%	90.1%
	d -7 to -5	11	-2.20 ± 0.32	0.01 ± 0.04	76	0.85	0.11	89.1%	9.1%	98.0%

Supplementary Table 7.4. Univariate models for clinical mastitis cases with no pathogen isolated for the 10 explanatory variables. For each model, the number of cases, intercept and slope estimates (± SE), corrected Akaike's Information Criterion (**AICc**), and the percentage of cases and controls identified correctly (accuracy), sensitivity (**Se**) and specificity (**Sp**) at the optimal cutoff are presented.

Explanatory Variable	Slope Range	N. cases	Intercept	Slope Estimate	AICc	*P* value	Cutoff	Accuracy (%)	Se (%)	Sp (%)
Fat %	d -7 to -1	83	-1.15 ± 0.41	-0.78 ± 1.31	355	0.56	0.18	56.8%	79.5%	48.5%
	d -7 to -2	81	-1.15 ± 0.43	-1.11 ± 1.15	344	0.34	0.30	59.5%	75.3%	53.6%
	d -7 to -3	77	-1.18 ± 0.44	-1.53 ± 0.82	326	0.06	0.22	58.8%	79.2%	51.4%
	d -7 to -4	74	-1.18 ± 0.43	-1.45 ± 0.65	315	0.03	0.28	61.3%	74.3%	56.7%
	d -7 to -5	64	-1.22 ± 0.39	-1.49 ± 0.60	281	0.01	0.19	56.8%	85.9%	47.2%
Lactose %	d -7 to -1	83	-1.16 ± 0.42	-1.90 ± 3.40	355	0.58	0.28	57.7%	75.9%	51.1%
	d -7 to -2	81	-1.15 ± 0.44	-5.14 ± 3.52	343	0.14	0.22	58.8%	76.5%	52.3%
	d -7 to -3	77	-1.16 ± 0.44	-0.63 ± 3.01	330	0.84	0.33	59.1%	75.3%	53.3%
	d -7 to -4	74	-1.15 ± 0.42	1.04 ± 2.32	320	0.66	0.32	61.0%	74.3%	56.3%
	d -7 to -5	64	-1.18 ± 0.38	-0.71 ± 2.07	288	0.73	0.29	59.1%	70.3%	55.4%
Protein %	d -7 to -1	83	-1.14 ± 0.41	-2.19 ± 3.26	355	0.50	0.29	58.4%	75.9%	52.0%
	d -7 to -2	81	-1.13 ± 0.42	-0.96 ± 3.19	345	0.76	0.33	58.8%	74.1%	53.2%
	d -7 to -3	77	-1.15 ± 0.43	-2.71 ± 2.63	329	0.30	0.29	59.8%	74.0%	54.7%
	d -7 to -4	74	-1.15 ± 0.42	-0.48 ± 1.93	320	0.81	0.18	58.5%	74.3%	52.9%
	d -7 to -5	64	-1.18 ± 0.38	-0.44 ± 1.56	288	0.78	0.18	58.4%	71.9%	53.9%
Conductivity	d -7 to -1	83	-1.14 ± 0.41	-0.03 ± 0.68	355	0.97	0.17	57.1%	75.9%	50.2%
	d -7 to -2	81	-1.14 ± 0.43	0.41 ± 0.60	345	0.49	0.20	58.3%	76.5%	51.6%
	d -7 to -3	77	-1.16 ± 0.44	0.09 ± 0.52	331	0.86	0.34	58.9%	75.3%	53.0%
	d -7 to -4	74	-1.18 ± 0.41	0.77 ± 0.44	318	0.08	0.31	65.7%	62.2%	67.0%
	d -7 to -5	64	-1.18 ± 0.38	0.26 ± 0.36	287	0.46	0.27	59.1%	70.3%	55.4%
Milk yield	d -7 to -1	83	-1.13 ± 0.41	0.04 ± 0.09	355	0.68	0.33	60.0%	75.9%	54.2%
	d -7 to -2	81	-1.13 ± 0.42	0.06 ± 0.09	345	0.55	0.29	58.6%	75.3%	52.5%
	d -7 to -3	77	-1.16 ± 0.44	0.04 ± 0.08	331	0.64	0.17	57.9%	77.9%	50.7%
	d -7 to -4	74	-1.16 ± 0.42	-0.01 ± 0.07	321	0.92	0.17	58.3%	74.3%	52.6%
	d -7 to -5	64	-1.18 ± 0.38	-0.08 ± 0.06	286	0.19	0.29	64.6%	65.6%	64.2%

LogSCC	d -7 to -1	83	-1.15 ± 0.41	0.04 ± 0.37	355	0.92	0.34	57.4%	75.9%	50.7%
	d -7 to -2	80	-1.15 ± 0.44	-0.07 ± 0.36	341	0.84	0.16	57.7%	78.8%	50.0%
	d -7 to -3	77	-1.15 ± 0.43	-0.16 ± 0.35	330	0.65	0.18	59.1%	77.9%	52.3%
	d -7 to -4	74	-1.15 ± 0.41	-0.07 ± 0.32	321	0.82	0.17	58.5%	77.0%	51.9%
	d -7 to -5	64	-1.17 ± 0.37	0.04 ± 0.26	287	0.88	0.32	61.2%	68.8%	58.6%
Activity	d -7 to -1	83	-1.19 ± 0.41	-0.04 ± 0.02	350	0.03	0.27	60.0%	78.3%	53.3%
	d -7 to -2	81	-1.19 ± 0.41	-0.04 ± 0.02	338	0.01	0.20	59.3%	81.5%	51.1%
	d -7 to -3	77	-1.19 ± 0.42	-0.03 ± 0.01	325	0.02	0.27	60.6%	76.6%	54.9%
	d -7 to -4	74	-1.18 ± 0.41	-0.02 ± 0.01	318	0.09	0.22	59.0%	77.0%	52.6%
	d -7 to -5	64	-1.19 ± 0.37	-0.01 ± 0.01	287	0.34	0.19	59.1%	75.0%	53.9%
Lying time	d -7 to -1	17	-1.76 ± 0.26	0.00 ± 0.01	101	0.70	0.17	84.5%	11.8%	97.0%
	d -7 to -2	17	-1.76 ± 0.26	0.00 ± 0.01	101	0.81	0.13	17.2%	100.0%	3.0%
	d -7 to -3	16	-1.81 ± 0.27	0.00 ± 0.01	97	0.93	0.14	50.0%	68.8%	46.9%
	d -7 to -4	16	-1.82 ± 0.28	0.00 ± 0.00	95	0.47	0.13	33.9%	87.5%	25.0%
	d -7 to -5	16	-1.79 ± 0.28	0.00 ± 0.00	95	0.39	0.13	39.1%	87.5%	30.9%
Lying bouts	d -7 to -1	17	-1.79 ± 0.27	-0.21 ± 0.32	100	0.52	0.12	20.7%	100.0%	7.1%
	d -7 to -2	17	-1.76 ± 0.26	0.06 ± 0.27	101	0.82	0.15	71.6%	47.1%	75.8%
	d -7 to -3	16	-1.81 ± 0.27	0.11 ± 0.20	96	0.56	0.15	74.6%	37.5%	80.6%
	d -7 to -4	16	-1.79 ± 0.27	0.18 ± 0.16	95	0.27	0.14	54.5%	62.5%	53.1%
	d -7 to -5	16	-1.82 ± 0.28	0.15 ± 0.12	94	0.20	0.18	80.9%	31.3%	89.4%
Lying bout duration	d -7 to -1	17	-1.75 ± 0.26	-0.03 ± 0.09	101	0.77	0.17	86.2%	5.9%	100.0%
	d -7 to -2	17	-1.73 ± 0.26	-0.05 ± 0.08	100	0.47	0.16	67.2%	41.2%	71.7%
	d -7 to -3	16	-1.82 ± 0.28	0.00 ± 0.06	97	0.96	0.14	50.0%	56.3%	49.0%
	d -7 to -4	16	-1.77 ± 0.27	-0.02 ± 0.04	96	0.65	0.17	85.7%	12.5%	97.9%
	d -7 to -5	16	-1.78 ± 0.27	-0.01 ± 0.03	95	0.66	0.18	85.5%	12.5%	97.9%