

Efficacy of On-Farm Programs for the Diagnosis and Selective Treatment of
Clinical and Subclinical Mastitis in Dairy Cattle

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DEDICATION

I would like to dedicate my thesis dissertation to my wife Noelia; parents Alfonso and Enedina; brother Oscar; grandparents Alfredo, Dolores, Estanislao and Leonor; uncle Pepe; aunts Maruja, Lola and Carmen; cousin Divina; and to the group of core friends from Spain and Madison. The love, education and support provided by my family and core friends make me not to be afraid in pursuing my goals and dreams.

ABSTRACT

The research reported in this dissertation includes two multi-state multi-herd clinical trials evaluating the efficacy of on-farm programs for the diagnosis and selective treatment of clinical and subclinical mastitis in dairy cattle.

The use of an OFC system for the selective treatment of clinical mastitis during lactation reduced intramammary antibiotic use by half and tended to reduce withholding time by one day, without significant differences in days to clinical cure, bacteriological cure risk, new infection risk and ICR risk (where the ICR risk represented the presence of infection risk, clinical mastitis risk, or removal from herd risk) within 21 days after the clinical mastitis event. Similarly, there were no differences between both treatment programs in long-term outcomes such as recurrence of clinical mastitis in the same quarter, somatic cell count, milk production, and cow survival for the rest of the lactation after the clinical mastitis event.

The treatment with intramammary Cephapirin Sodium of cows and quarters based on CMT results alone, or sequential testing using OFC to diagnose Gram-positives in CMT-positive quarters resulted in a higher bacteriological cure risk and reduced the ICR risk within 21 days after enrollment (significantly and only numerically, respectively for treatment each program). The implementation of both treatment programs required the administration of intramammary treatment and extended the time that milk is withhold

from the market. Both programs resulted in a significantly lower clinical mastitis risk and lower milk SCC during lactation (significantly and only numerically, respectively for each treatment program). However, the implementation of both treatment programs did not result in higher milk production, improved reproductive performance or lower risk for removal from the herd.

A secondary objective of both clinical trials was to validate the use of the Minnesota Easy Culture Bi-Plate System. This OFC system is a useful cow-side test to correctly identify bacterial growth, Gram-positive bacterial growth, or Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases and in CMT-positive quarter milk samples collected after parturition. Treatment decisions based on identification of bacterial growth, or Gram-positive bacterial growth specifically, were correct over 73% of the time.

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CHAPTER I

LITERATURE REVIEW: EPIDEMIOLOGY OF, AND TREATMENT CONSIDERATIONS FOR, CLINICAL MASTITIS DURING LACTATION AND SUBCLINICAL MASTITIS AFTER PARTURITION

Mastitis in dairy cattle has significant ramifications, including financial losses to dairy farmers, adverse effects on cow welfare and potential influences on public health. The following review will discuss epidemiology, economics, and treatment of clinical mastitis during lactation and subclinical mastitis after parturition. It will also justify the need to develop and validate new tools to aid in the diagnosis and guide strategic treatment of clinical and subclinical mastitis on-farm, and promote judicious use of antibiotics.

EPIDEMIOLOGICAL CLASSIFICATION OF MASTITIS PATHOGENS

Bovine mastitis, defined as inflammation of the mammary gland, can have an infectious or noninfectious etiology. Organisms as diverse as bacteria, *Mycoplasma*, yeasts and algae have been implicated as causes of the disease; Watts (1988) identified 137 different organisms as a cause of mastitis. The vast majority of mastitis is of bacterial origin.

Staphylococcus spp., *Streptococcus* spp., and coliforms account for more than 90% of all

bacterial isolates from mastitis cases (Riekerink *et al.*, 2007; Sargeant *et al.*, 1998; Erskine *et al.*, 1988).

Historically, mastitis pathogens have been classified as either “contagious” or “environmental” (Bramley and Dodd, 1984; Smith *et al.*, 1985; Fox and Gay, 1993). The contagious pathogens are considered as organisms adapted to survive within the host, in particular within the mammary gland, and are typically spread from cow to cow, at or around the time of milking. The most common contagious pathogens are *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma* spp. In contrast, the environmental pathogens are opportunistic invaders of the mammary gland, not especially adapted to survival within the host; typically they enter, multiply, illicit a host immune response and are eliminated. The primary source of environmental pathogens is the surrounding environment (e.g. contaminated bedding, feces, water) in which a cow lives. The most common environmental pathogens are *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Serratia*, *Pseudomonas*, *Proteus*, *Enterococcus*, *Streptococcus uberis* and *Streptococcus dysgalactiae*.

The line between classic contagious and environmental behaviour of mastitis pathogens has become blurred. Persistent infection with both *Streptococcus uberis* (Todhunter *et al.*, 1995; Zadoks, 2003) and *E. coli* (Hill *et al.*, 1979; Lam *et al.*, 1996; Dopfer *et al.*, 1999; Bradley and Green, 2001) has been reported. Studies using DNA fingerprinting have stated that 9.1% (Lam *et al.*, 1996), 4.8% (Dopfer *et al.*, 1999), and 20.5% (Bradley and Green, 2001) of clinical *E. coli* mastitis recurred in a quarter. The persistence of

infections, and the proportion of clinical *E. coli* cases occurring in different quarters of the same cow caused by a genotype previously identified in that cow, may suggest transmission between quarters in a manner more commonly associated with contagious pathogens (Bradley and Green, 2001). The same contagious behavior for an ‘environmental’ pathogen has been proposed for *Streptococcus uberis* infections (Zadoks *et al.*, 2003). Epidemiological and molecular data suggest infection from environmental sources with a variety of *Streptococcus uberis* strains, as well as within-cow and between-cow transmission of a limited number of *Streptococcus uberis* strains, with possible transfer of bacteria via the milking machine.

Pathogenesis and Pathophysiology of Mastitis Pathogens

Gram staining, empirically developed by Christian Gram in the late 1800's, has become an important means of classification in regards to bacterial mastitis since classifies bacteria based upon their cellular structure. The cell wall structure of pathogens plays a key role in the response of the cow, as well as in the infection pathogenesis and pathophysiology. Cell wall structure also has significant implications for antibiotic treatment selection.

Gram-Positive Bacteria

The Gram-positive bacterial cell wall is composed almost entirely of peptidoglycan layers, a relatively complex polymer of sugars with amino acid linkages (as reviewed by

Navarre and Schneewind, 1999). The thick peptidoglycan layer allows Gram-positive bacteria the ability to withhold crystal violet stain. Uniquely, this group of bacteria often contains teichoic acid which is incorporated within the peptidoglycan. Some of the most common Gram-positive pathogens include *Staphylococcus aureus*, coagulase-negative staphylococcus, *Enterococcus*, *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus dysgalactiae*.

Staphylococcus aureus

Infection with *Staphylococcus aureus*, the most infectious of the staphylococcal pathogens, is often referred to as contagious mastitis because it is commonly spread from infected cows to other noninfected cows at milking (Mellenberger *et al.*, 1994; Nickerson 1993). The colonization of the mammary gland by *Staphylococcus aureus* usually results in a chronic subclinical infection, although it also can alternate with clinical mastitis episodes. Less frequently *Staphylococcus aureus* infections result in a peracute infection developing a gangrenous mastitis (Anderson, 1982).

To establish an infection, these organisms colonize the skin and streak canal and attach to epithelial cell surfaces to breach this first line of defense (Nickerson, 1987; Nickerson, 1993). Once within the mammary gland, *Staphylococcus aureus* produces hemolysins that damage tissue, leading to intracellular colonization by the organisms (Anderson, 1982; Gudding *et al.*, 1983; Nickerson *et al.*, 1981). These infections can become chronic because of their intracellular location, making it more difficult for the immune system to

recognize and eliminate the bacteria. The second line of defense is the immune system that includes leukocytes in the teat ducts and in the gland. These polymorphonuclear neutrophils are efficient at removing bacteria that have invaded the gland. However, *Staphylococcus aureus* possesses components that allow it to escape phagocytosis and intracellular killing (White *et al.*, 1980; Harmon *et al.*, 1982; Craven *et al.*, 1984). This mechanism may account for the pathogen's apparent resistance when antibiotic agents selected on the basis of in vitro sensitivity are used, because commercial mastitis therapies do not reach intracellular pathogens (Fox and Gay, 1993).

Coagulase-negative staphylococcus

Coagulase-negative staphylococci (CNS) are often considered pathogens of minor importance, especially in contrast to *Staphylococcus aureus*, streptococci, and coliforms, which may cause severe mastitis. A number of CNS species, identified with methods based on phenotype, have been isolated from bovine mastitis. The two species isolated most often are *Staphylococcus chromogenes* and *Staphylococcus simulans* (Jarp, 1991; Taponen, 2007), but also *Staphylococcus hyicus* and *Staphylococcus epidermidis* have frequently been reported (Waage *et al.*, 1999; Rajala-Schultz, 2004).

These bacteria usually cause subclinical or mild clinical mastitis, but have also been reported to produce severe cases of mastitis (Jarp, 1991). In a recent Finnish study, half of the cases were clinical, but in majority of the clinical cases the signs were very mild (Taponen *et al.*, 2006). No significant differences in the severity of clinical signs caused

by the two most common CNS species were found, which agrees with previous studies (Jarp, 1991). Coagulase-negative staphylococcus infections are generally associated with an increase in somatic cell count (SCC) in the infected quarter (Djabri *et al.*, 2002; Taponen *et al.*, 2007). Mastitis caused by CNS may result in a slight decrease in milk production (Gröhn *et al.*, 2004; De Vliegher *et al.*, 2005). Gröhn *et al.* (2004) have shown that multiparous cows with clinical CNS mastitis were, before the onset of mastitis, higher producers than control cows without CNS mastitis, suggesting that milk production losses associated with CNS infection may have been previously underestimated.

Spontaneous elimination (cure) of CNS mastitis is generally regarded as a common occurrence. Some studies have shown spontaneous elimination rates of about 60-70% (McDougall, 1998; Wilson *et al.*, 1999). However, markedly lower rates, 15%-44%, have also been reported (Rainard and Poutrel, 1982; Timms and Schultz, 1987, Deluyker *et al.*, 2005). Certain common CNS species may be capable of persisting in the mammary gland for months or even throughout the lactation period (Laevens *et al.*, 1997; Aarestrup *et al.*, 1999; Chaffer *et al.*, 1999). A recent study from Finland showed that half of the CNS infections detected post partum persisted until the end of lactation and caused elevated SCC during the entire lactation (Taponen *et al.*, 2007).

Streptococcus agalactiae

Streptococcus agalactiae is a highly contagious obligate parasite of the bovine mammary gland (McDonald, 1977). It generally causes a low-grade persistent type of infection and does not have a high self-cure rate. Unidentified infected cattle function as reservoirs of infection, because they are not selected for treatment, segregation or culling (Farnsworth, 1987).

Streptococcus agalactiae has the ability to adhere to the mammary tissue of cows and the specific microenvironment of the bovine udder is necessary for the growth of the bacterium (Wanger and Dunny, 1984). For an obligate intramammary (IMM) pathogen like *S. agalactiae*, the bovine udder is recognized as the only reasonable source of the organism in the milk. Consequently, isolates in the bulk tank are usually assumed to have come from the udder (Bartlett *et al.*, 1991; Gonzalez *et al.*, 1986).

Non -agalactiae streptococci

Common environmental streptococci include species of streptococci other than *Streptococcus agalactiae* and species of enterococci. For *Streptococcus dysgalactiae* and *Streptococcus uberis*, there is disagreement with respect to their classification. In some laboratories, the two species are grouped together as ‘environmental streptococci’ (Todhunter *et al.*, 1995; Wilson *et al.*, 1997). However, *Streptococcus dysgalactiae* and *Streptococcus uberis* differ in many bacteriological and epidemiological characteristics (Barkema *et al.*, 1999; Leigh *et al.*, 1999; Vieira *et al.*, 1998). *Enterococcus* spp. have commonly been included in the heterogeneous grouping of non-agalactiae streptococci.

Exposure of uninfected glands to environmental streptococci occurs during milking, between milkings, during the dry period, and prior to parturition in first lactation heifers. In one seven-year study that reported the dynamics of environmental streptococcal mastitis in an experimental herd, the dry period was identified as the time of greatest susceptibility to new environmental streptococcal intramammary infections (IMI) (Todhunter *et al.*, 1985). The epidemiology of IMI caused by *Enterococcus* spp. is relatively undefined with regards to common farm management practices that may lead to the control of mastitis caused by these organisms.

Approximately one-half of environmental streptococcal IMI cause clinical mastitis during lactation (Todhunter *et al.*, 1985). Severity of clinical signs is generally limited to local inflammation of the gland. A total of 43% of clinical cases had signs limited to abnormal milk (mild), 49% involved abnormal milk and swollen gland (moderate), and only 8% involved systemic signs such as fever and anorexia (severe). During lactation, the incidence of clinical mastitis was greatest the first week after calving and decreased throughout the first 305 days in milk (Hogan *et al.*, 1989).

It has been reported that environmental streptococcal IMI tend to be short duration infections with only a relatively few becoming chronic (Todhunter *et al.*, 1985). However, a more recent study reported that half of the observed infections lasted more than an estimated 42 days and approximately one in four infected episodes lasted more than 72 days, emphasizing that chronic infections are no exception (Zadoks *et al.*, 2003).

Gram-Negative Bacteria

Gram-negative bacteria tend to have a more complex layering in their cell wall structure.

While the cell wall does contain peptidoglycans, it also contains a complex and species unique, lipopolysaccharide layer (LPS) (as reviewed by Beveridge, 1999).

Lipopolysaccharide, or endotoxin, typically elicits an acute immune response in an infected animal. *Escherichia coli* is perhaps the primary Gram-negative contributor to mastitis in dairy cows.

The term 'coliform mastitis' frequently is used incorrectly to identify mammary disease caused by all Gram-negative bacteria. Genera classified as coliforms are *Escherichia*, *Klebsiella*, and *Enterobacter*. Other Gram-negative bacteria frequently isolated from IMIs include species of *Serratia*, *Pseudomonas*, and *Proteus*.

Gram-negative bacteria are the etiological agents most often isolated from acute clinical cases of mastitis. The severity of clinical cases caused by coliform bacteria ranges from mild local signs to severe systemic involvement. The vast majority of clinical coliform cases are characterized by abnormal milk and a swollen gland. Only about 10% of clinical coliform cases result in systemic signs including fever, anorexia, and altered respiration (Hogan *et al.*, 1989; Smith *et al.*, 1985).

Coliform bacteria do not appear to colonize inside the mammary gland, but multiply in the secretion without attachment to epithelial tissue (Frost *et al.*, 1977; Opdebeeck *et al.*, 1988). The primary cellular defense of the bovine mammary gland against coliform mastitis is the phagocytosis and killing of bacteria by neutrophils (Hill, 1981; Van Werben, 1997). The peak bacterial numbers in the gland and clinical severity of disease are often dependent on the speed and efficiency of the neutrophil response. The ability of a strain to evade neutrophils is a key virulence factor for coliform bacteria.

Endotoxin, the lipopolysaccharide portion of the Gram-negative bacterial wall, is the primary virulence factor of Gram-negative bacteria responsible for damage to the cow. Endotoxin is released from the bacteria at the time of cell death initiating an inflammatory response. Locally, endotoxin does not directly affect secretory cells, but disrupts the blood flow (Shuster *et al.*, 1991). Decreased milk production during clinical coliform mastitis results both directly and indirectly from the local and systemic effects of endotoxin (Hirboen *et al.*, 1999; Hoeben *et al.*, 2000). It was reported that about 45% of the severe cases of coliform mastitis result in bacteremia and septicemia as the blood-milk barrier is destroyed (Wenz *et al.*, 2001).

Incidence of coliform IMI during lactation is highest at calving and decreases as days in milk advances. The average duration of *E. coli* IMI during lactation is less than ten days (Todhunter *et al.*, 1991). Duration of IMI caused by *Klebsiella pneumoniae* average about 21 days (Smith *et al.*, 1985). Chronic infections of greater than 90 days caused by *Escherichia coli* or *Klebsiella pneumoniae* are relatively rare. A major difference between

IMIs caused by coliform bacteria and those caused by other Gram-negative bacteria is the duration that bacteria persist in the mammary gland. IMIs caused by *Serratia* spp. and *Pseudomonas* spp. often are chronic infections that may persist multiple lactations (Hogan *et al.*, 1989).

CLINICAL MASTITIS IN LACTATING COWS

Incidence and Etiology of Clinical Mastitis

Clinical mastitis clinical is defined as the inflammation of the mammary gland accompanied by secretion of abnormal milk, some times in combination of a swelling udder, and a few times also combined with a systemically sick animal. Quantitative information on the incidence and etiology of clinical mastitis in North America is scarce in comparison with European countries. Three North American studies reported this information from non-randomly selected dairy herds from nationwide Canada, Ontario and Pennsylvania respectively (Riekerink *et al.*, 2007; Sargeant *et al.*, 1998; Erskine *et al.*, 1988) (Table 1.1). A 22.4% lactational risk and 20.4 cases per 100 cow-years incidence were reported in the Canadian studies. The mean incidence of clinical mastitis in herds with low SCC was 4.23 cases / 100 cows / month and for high SCC herds 2.91 cases / 100 cows / month in the USA study. In a recent report from Wisconsin where herds were investigated due to mastitis problems, the clinical mastitis incidence reported was of 48.7 cases / 100 cows / year (Cook and Mentik, 2006).

In the Ontario study, representing the most recent study in the Great Lakes region, the bacteria isolated from clinical mastitis cases were *Staphylococcus aureus* (6.7%), *Streptococcus agalactiae* (0.7%), other *Streptococcus* spp. (14.1%), coliforms (17.2%), Gram-positive bacilli (5.5%), *Corynebacterium bovis* (1.7%), and CNS (28.7%). There was no bacterial growth in 17.7% of samples, and 8.3% of samples were contaminated. It has been reported that there is a shift towards environmental pathogens as the major causes of clinical mastitis in the USA, Canada and several European Countries (Anon, 2001; Green and Bradley, 1998). However, there are significant differences in the etiology of clinical mastitis cases among those countries. Even within a country, there are differences in the bacteria isolated in the different regions, due to climate and dairy management differences (Guterbock *et al.*, 1993).

Antibiotic Therapy Efficacy for Mastitis Pathogens

Gram-Positive Bacteria

The Gram-positive bacterial cell wall is composed almost entirely of peptidoglycan layers (as reviewed by Navarre and Schneewind, 1999). Many of these infections are sensitive to antibiotics available to administer by the IMM route in lactating cows, β -lactam antibiotics, since they inhibit cell wall synthesis by targeting peptidoglycans.

A retrospective cohort study in New York determined that IMM therapy was not beneficial for clinical mastitis caused by most pathogens other than streptococci (Wilson

et al., 1999). In another experimental study *Streptococcus uberis* induced infections benefited from administration of IMM therapy compared with infections treated with oxytocin only (Hillerton and Semmens, 1999). Similarly, field studies have found that IMM antibiotic therapy was beneficial for Gram-positive organisms such as Streptococci and coagulase-negative staphylococci, but ineffective for Gram-negative (Hallberg *et al.*, 1994; Roberson *et al.*, 2004). In a clinical trial in three Californian dairies, bacteriologic cure assessed at 4 and 20 days after treatment with amoxicillin, cephalosporin, or oxytocin (no antibacterial) did not differ for mild clinical mastitis cases caused by any pathogen, although antibacterial treatment resulted in better clinical cure rates for cases caused by pathogens other than streptococci and coliforms (Guterbock *et al.*, 1993). A study conducted at the University of Illinois dairy herd compared antibiotic administration in conjunction with supportive measures versus supportive measures alone for treatment of clinical mastitis. The authors reported that when mastitis was caused by *Streptococcus* spp. or coliform bacteria, clinical cure rate by the tenth milking was significantly greater if antibiotics were used, and bacteriologic cure rate at 14 days was significantly greater when antibiotics were used, particularly if mastitis was caused by *Streptococcus* spp. (Morin *et al.*, 1998). An economic analysis of California data, however, determined that although milk production and survival in the herd did not differ between antibacterial-treated and non-antibacterial-treated cows, the rate of both relapses and recurring cases was higher in non-antibacterial-treated cows, especially among streptococcal cases (Van Eenennaam *et al.*, 1995). A case report from a Colorado dairy also reported an acute increase in incidence of clinical mastitis, prevalence of IMI, and subsequent increase in

herd somatic cell count associated with streptococcal IMI following adoption of a non-antibiotic approach to treat clinical mastitis (Cattell, 1996).

Staphylococcus aureus mastitis poses difficult therapeutic problems because of several exposed pathogenesis and pathophysiology factors. Reported cure rates for *Staphylococcus aureus* mastitis vary considerably. The probability of cure depends on cow, pathogen, and treatment factors. Cure rates decrease with increasing age of the cow, increasing somatic cell count, increasing duration of infection, increasing bacterial colony counts in milk before treatment, and increasing number of quarters infected. The most important treatment factor affecting cure is treatment duration. Increased duration of treatment is associated with increased chance of cure (Barkema *et al.*, 2006).

Gram-Negative Bacteria

Gram-negative bacteria tend to have a more complex layering in their cell wall structure. While the cell wall does contain peptidoglycans, it also contains a complex and species unique, lipopolysaccharide layer (LPS) (as reviewed by Beveridge, 1999).

Lipopolysaccharide, or endotoxin, typically elicits an acute immune response in an infected animal. *Escherichia coli* is perhaps the primary Gram-negative contributor to mastitis in dairy cows. Because many antibiotics target peptidoglycans, treatment of Gram-negative bacteria proves difficult in comparison to Gram-positive pathogens (Pyörälä *et al.*, 1994).

The efficacy of antibiotics was also questioned on the basis of knowledge of the pathophysiology of coliform mastitis (Pyörälä *et al.*, 1994; Erskine *et al.*, 1992), which includes the spontaneous rapid drop of milk bacterial counts 8 to 24 h after infection and the risk of a massive release of bacterial endotoxins induced by antimicrobials (Hill *et al.*, 1978; Pyörälä *et al.*, 1994; Shenep and Mogan, 1984; Shenep *et al.*, 1985). Clinical recognition of coliform mastitis usually occurs after peak bacterial numbers have been attained (Hill *et al.*, 1979; Anderson *et al.*, 1985; Erskine *et al.*, 1992). Thus, by the time therapy is initiated, maximal release of endotoxin has likely occurred, which raises concerns regarding the advantages of antibacterial therapy in alleviating the effects of acute coliform mastitis. *Klebsiella* spp. infections last significantly longer than *E. coli* infections and are not likely to respond to antibiotic treatment (Smith *et al.*, 1985; Roberson *et al.*, 2004).

Field trials and trials with experimentally induced coliform mastitis have failed to prove the efficacy of antimicrobial treatment. In a retrospective cohort study in bacteriological cure rates for untreated cases of *E. coli* and *Klebsiella* spp. were high, 85% (Wilson *et al.*, 1999). Cows experimentally challenged with *E. coli* and dosed with 500 mg of IMM gentamicin q 14 hrs did not have lower peak bacterial concentrations in milk, duration of infection, convalescent somatic cell or serum albumin concentrations in milk, or rectal temperatures, as compared to untreated challenged cows (Erskine *et al.*, 1992). In a Californian clinical trial, bacteriologic cure and clinical cure did not differ after treatment with amoxicillin, cephalosporin, or oxytocin (non-antibacterial) for mild clinical mastitis cases caused by coliforms (Guterbock *et al.*, 1993). Another field trial intended to

determine the efficacy of 4 methods (IMM amoxicillin, frequent milkout, a combined IMM amoxicillin and frequent milk-out, and no treatment) for managing mild to moderate clinical mastitis in a university dairy herd. Treatment method appeared to have little effect on clinical and microbiological cures, milk production, disease progression, and California Mastitis Tests scores for *E. coli* mastitis, as nearly all cases recovered within a short time frame (Roberson *et al.*, 1994). Similarly, one other field study found that IMM antibiotic therapy was ineffective for Gram-negative intrammary infections (Hallberg *et al.*, 1994). The controversy over the use of antimicrobial treatment for coliform mastitis is further heightened by a controlled experiment at the University of Illinois dairy herd, showing that clinical and bacteriological cure rates were significantly higher in clinical mastitis cases caused by environmental streptococci or coliform bacteria when treated by IMM administration of cephalosporin and/or intravenous administration of oxytetracycline (Morin *et al.*, 1998). The interpretation of that study is problematic, however, because data from two very different bacteriological groups, streptococci and coliforms, had been pooled.

Economics of Clinical Mastitis Therapy

The 2005 Bulletin of the International Dairy Federation (IDF) evaluating the economic cost of mastitis estimates a € 270.33 cost for a clinical case of mastitis when treated immediately after detection. The IDF classifies the total cost of clinical mastitis on five main fractions: a) extra labor cost; b) losses due to decreased milk quality; c) losses due to less efficient milk production from chronic subclinically infected cows; d) losses due

to discarded milk, costs of antibiotics for treatment, and veterinary fees; and e) losses due to increased replacement rate and/or culling of cows at sub-optimal time in lactation. The loss due to therapy is very obvious and visible, and consists of value of discharged milk, value of fed milk minus saved calf feed, veterinary fees, cost of antibiotics or other therapeutics, and extra labor due to therapy.

In a study where the cost of clinical mastitis was estimated to be greater than \$100/case and averaged \$40 to \$50 per cow in herd per year, decreased milk production and milk withheld from the market were reported as the main economic losses (\$90 per case, which was 85% of the estimated losses) (Hoblet *et al.*, 1991). Discarded milk following treatment may account for as much as 73% of lost marketable milk, and in herds that do not have a judicious treatment program, losses from discarded milk alone can exceed \$100 per cow in the herd per year (Bartlett, 1991). In addition, economic loss due to discarded milk may be comparable with the loss caused by decreased milk production. There is a difference, however, in that discarded milk is produced by the cows, which means that feeding costs for that amount of milk have to be taken into account with the calculations. Thus, there is increased awareness among producers of treatment-related costs and the economic costs of extensive antibacterial therapy for mastitis (Erskine *et al.*, 2003).

Contrary to the expected reduction in discarded milk in a non-antibiotic treatment regimen, a clinical trial evaluating two antibiotic treatment regimens and one based just in the administration of oxytocin found that the cost of treatment, calculated by adding

the cost of the therapy to the value of the milk withheld, did not differ between one of the antibiotic treatments and the non-antibiotic regimen. Treatment costs per episode of clinical mastitis were as follows: \$54.47 when 62.5 mg of IMM amoxicillin were administered every 12 h for three milkings with a 96 h milk withdraw; \$38.53 when 200 mg of IMM cephalosporin were administered every 12 h for two milkings with a 60 h milk withdraw; and \$34.88 when 100 U of intramuscular oxytocin were administered every 12 h for three milkings and no milk withdraw. The oxytocin treatment costs were not significantly lower than for amoxicillin because some of the affected quarters in the former group required an increased number of milkings before milk returned to normal appearance (Van Eenennaam *et al.*, 1995). However, authors recognized that the milk withhold costs for the cows in the oxytocin group included milkings during which cows were producing grossly normal milk following their recovery from mastitis, but those cows remained in the hospital string to allow sample collection along with their contemporary antibiotic treatment group. In this study, there was no treatment effect on total milk production, fat production, or time to removal of the enrolled cows from the herd.

In a study where cows with clinical mastitis were given either antibiotics in addition to supportive treatment, or supportive treatment alone, a cost analysis that included milk loss and treatment costs was performed (Shim *et al.*, 2004). Cows with clinical mastitis that were given only supportive treatment lost 230 ± 172 kg more milk and incurred $\$94 \pm 51$ more cost per lactation than cows given antibiotics and supportive treatment. In order to calculate total milk losses per lactation the actual amount of discarded milk was

added to milk yield loss. Furthermore, cows given only supportive treatment showed a response pattern of 305-d milk yield loss and economic loss per lactation that varied 2 to 3 times as much as cows treated with antibiotics.

Previous studies were able to quantify the economic impact of clinical mastitis treatment associated costs. However, the potential reduction in treatment costs and discarded milk of a non antibiotic treatment regimen have not yet been fully captured.

To summarize, it has been reported that more than half of cultures from clinical mastitis cases yield no growth or are Gram-negatives. These cases of clinical mastitis may not benefit from IMM antibiotic therapy. Conversely, Gram-positive IMI benefit from IMM therapy. The selective treatment of clinical mastitis might reduce treatment related costs, and promote judicious use of antibiotics.

SUBCLINICAL INTRAMAMMARY INFECTIONS IN COWS AFTER PARTURITION

Dry Period Intramammary Infections Dynamics

The importance of the dry period in the control of contagious mastitis has been recognized for more than 50 years (Neave *et al.*, 1950). Many contagious mastitis infections (especially infections caused by *Staphylococcus aureus* and *Streptococcus agalactiae*) are subclinical. The use of dry cow therapy is a well-established and cost-

efficient method of eliminating subclinical mastitis infections. The importance of the dry period in the control of environmental mastitis has been more recently recognized.

Researchers in the United States have found that of all new intramammary Gram-negative infections, 61% occurred during the dry period. Environmental Gram-positive bacteria also infect the udder during the non-lactating period (Todhunter *et al.*, 1991). Those investigators also reported that 50.5% of new IMI with environmental streptococci occurred in the non-lactating udder - a rate of new infection 5.5 times greater than during lactation (Todhunter *et al.*, 1995).

Studies in the United Kingdom investigating the significance of IMI with these major pathogens during the non-lactating period highlighted the importance of considering new infections before calving. They quantitatively assessed the impact of dry-period IMI on clinical mastitis during the next lactation. Intramammary infections at, and during the dry and immediate post-calving period, increase the risk of clinical mastitis in the next lactation. They reported that 55.6% of clinical mastitis cases due to *Streptococcus uberis* and 33.3% of cases due to *Streptococcus dysgalactiae* were caused by new infections originally acquired during the dry period. This mastitis occurs at a greater rate after calving than mastitis not associated with dry period infections (Bradley and Green, 2000; Bradley and Green, 2001; Green *et al.*, 2002).

In one study DNA fingerprinting of enterobacterial strains was used (Bradley and Green, 2000). Of all the coliform mastitis events monitored during the first 100 d of lactation,

52.6% resulted from an infection originally acquired during the previous non-lactating period. Another study reported that over 60% of clinical mastitis events in quarters in which the same pathogen was identified during the dry period, occurred within two weeks of calving, and 90% occurred within 150 days of calving (Green *et al.*, 2002). This was in contrast to the approximately constant rate of clinical mastitis during lactation that occurred in quarters from which the same pathogens were not cultured during the dry period. It was concluded that the pattern and rate of clinical mastitis over lactation on a dairy farm can give an indication of the impact of dry period IMI on clinical mastitis and moreover, the areas to target preventive measures.

Prevalence and Etiology

Reported prevalence of quarter IMI at parturition in North-American studies ranges from 29% to 63% (Fox *et al.*, 1994; Roberson *et al.*, 1994; Kirk *et al.*, 1996; Oliver *et al.*, 1997; Sargeant *et al.*, 2001; Godden *et al.*, 2003; Wallace *et al.*, 2004). At the cow level, Sargeant *et al.* (2001) reported a prevalence of infection of 71%. In that study 25.2, 26.0, 13.7, and 6.1% of the infected cows had 1, 2, 3, and 4 quarters infected, respectively. The same prevalence of infection was reported in a study where composite quarter milk samples only from heifers were collected after parturition (Kirk *et al.*, 1996). A lower prevalence of infection was reported by Roberson *et al.* (1994), with 55% of the heifers and 44% of the cows infected in the first 4 days after calving. Coagulase-negative staphylococci and environmental streptococci are the most common culture results at

parturition. Intramammary infection prevalence and etiology for the previously cited studies are reported in Table 1.2.

Antibiotic Therapy Efficacy

Controlled studies evaluating the efficacy of antibiotic therapy for IMIs early in lactation are scarce and few, with the ones carried out reporting mixed results. Rosenberg *et al.* (2002) evaluated the response to therapy with IMM cephapirin sodium on California Mastitis Test (CMT) positive quarters in fresh cows on cure rates and SCC. It was determined that by the 4-week post-calving evaluation, quarters treated with cephapirin sodium had significantly increased cure rates, and somatic cell counts were significantly reduced, as compared with untreated control quarters. Conversely, Wallace *et al.* (2004) also randomly assigning cows with CMT-positive quarters to receive either IMM cephapirin sodium or no treatment, found that there was no difference in cure rates for IMM antibiotic-treated quarters for major pathogens compared to the untreated controls. However, there was an advantage for cure rates using antibiotics against environmental streptococcal infections. Quarters with streptococci infections were 3.5 times more likely to cure if treated with sodium cephapirin.

Similarly, the efficacy of antibiotic therapy for treating IMIs in the immediate prepartum period is also controversial. A series of trials evaluating the efficacy of prepartum IMM antibiotic therapy on mastitis in heifers concluded that prepartum IMM antibiotic infusion of heifer mammary glands at 7 or 14 d before expected parturition was an effective procedure for eliminating many infections in heifers during late gestation, and

for reducing the prevalence of mastitis in heifers during early lactation and throughout lactation (Oliver *et al.*, 1992; Oliver *et al.*, 1997; Oliver *et al.*, 2003; Oliver *et al.*, 2004). Prepartum antibiotic-treated heifers had a lower prevalence of mastitis pathogens isolation throughout lactation, produced significantly more milk than control heifers and had significantly lower somatic cell count scores than untreated control heifers (Oliver *et al.*, 2003). In this study, prepartum antibiotic-treated heifers produced 531 kg more milk than heifers in the untreated control group. Conversely, a multi-state, multi-herd study reported that, although prepartum IMM antibiotic therapy did reduce the number of heifer IMI postpartum, milk production, SCC, and reproductive performance during the first 200 d of the first lactation were not significantly affected by treatment (Borm *et al.*, 2006).

Economic Impact

A Belgian study used monthly DHIA data to study the relationship between elevated somatic cell count (SCC) measured in the first 2 wk after calving and SCC in subsequent months of the first lactation. Elevated SCC in early lactation was associated with elevated test-day SCC, and a higher probability of test-day SCC exceeding 200,000 cells/ml (De Vliegher *et al.*, 2004). Elevated SCC in early lactation in heifers was associated with a decrease in lactation milk production (De Vliegher *et al.*, 2005a). In this study there was also a positive association between SCC of dairy heifers in early lactation and the culling hazard during the first lactation (De Vliegher *et al.*, 2005b). For each unit increase in the log-transformed SCC in early lactation, the culling hazard increased by 11%. Elevated

SCC in early lactation was also found to increase the probability of clinical mastitis over the first lactation (Rupp and Boichard, 2000). Conversely, in a study conducted in a single Californian dairy, environmental streptococcal and coagulase-negative staphylococcal infections in early lactation had no relationship with SCC or milk production in first-lactation heifers (Kirk *et al.*, 1996).

The relationship between subclinical mastitis defined by milk culture during early lactation and subsequent reproductive performance in Jersey cows was evaluated at the University of Tennessee dairy herd. Cows with subclinical mastitis before first insemination had increased days to first breeding service (74.8 ± 2.7 d), increased days open (107.7 ± 6.9 d), and increased services per conception (2.1 ± 0.2) as compared with controls (67.8 ± 2.2 d, 85.4 ± 5.8 d, 1.6 ± 0.2). Subclinical mastitis followed by clinical mastitis resulted in the most severe loss in reproductive performance (Schrick *et al.*, 2000).

To summarize, subclinical mastitis IMI at calving is highly prevalent, and has important consequences in cow health and production throughout the future lactation. The successful identification and treatment of those infections immediately after parturition before milk is saleable, has the potential to diminish the disease economical impact, while treatment related costs are reduced. However, similarly to clinical mastitis, treatment may only benefit Gram-positive IMIs.

ANTIBIOTIC USAGE ON DAIRY FARMS

Antibiotic Usage Rates

Antibiotics are used largely for three purposes in animals: therapeutic use to treat sick animals, prophylactic use to prevent infection in animals and as growth promoters to improve feed utilization and production. In the United States, a limited number of antimicrobial drugs are marketed for IMM treatment of mastitis. Antimicrobial classes include β -lactams (penicillin, cephalosporins, ceftiofur, amoxicillin, ticarcillin, and cloxacillin), macrolides (erythromycin), coumarins (novobiocin), and lincosamides (clindamycin) (FDA–Center for Veterinary Medicine, 2007).

Mastitis has been recognized as the most frequent reason for antibiotic use in lactating dairy cattle (Sundlof *et al.*, 1995; Mitchell *et al.*, 1998). However, those studies did not quantify the density of use. In a recent study in 20 Wisconsin conventional dairies, 80% of all antimicrobial drugs used were used for treatment or prevention of mastitis (IMM compounds for clinical mastitis, 38%; parenteral compound for clinical mastitis, 17%; dry cow therapy, 28%). Parenteral antimicrobial drugs used for the treatment of mastitis accounted for about half of the parenteral usage and 17% of the total usage in adult cows (Pol and Ruegg, 2007). In order to estimate antimicrobial drug exposure at the farm level, the authors characterized a veterinary drug defined daily dose (DDD) as the maximum dose that a standard animal (BW = 680 kg) would receive if it were treated following the FDA-approved label dosages. Density of antimicrobial drug usage was expressed as the number of DDD per adult cow per year. On conventional farms, the estimated overall

exposure to antimicrobial drugs was 5.43 DDD per cow per year, composed of 3.58 and 1.85 DDD of IMM and parenteral antimicrobial drugs, respectively. Of total IMM antimicrobial drug usage, treatment of clinical mastitis contributed 2.02 DDD compared with 1.56 DDD attributed to the use of dry cow therapy. Of total parenteral treatments, the distribution of exposure was 0.52 (dry cow therapy), 1.43 (clinical mastitis treatment), 0.39 (treatment of foot disease), 0.14 (treatment of respiratory disease), and 0.32 (treatment of metritis) DDD. For treatments of foot infections (0.33 DDD), respiratory infections (0.07 DDD), and metritis (0.19 DDD), the mean density of ceftiofur usage was significantly greater compared with other compounds.

Public Health Concerns

Problems attributed to the use of antibiotics in food producing animals include those of antibiotic residues and the potential for development of antibiotic resistance.

Until relatively recently, controls on antibiotic use in animals focused almost exclusively on the control of residues in the tissues of treated animals. Concerns about residues revolve around allergic reactions and the possible adverse effects on the flora of the human gastrointestinal tract by selecting for resistance or transfer of resistance (Barton, 2000). Data for 1994 through 1997 from a large milk marketing cooperative that operated in multiple states throughout the northeastern and midwestern United States revealed that violative antimicrobial residues were detected at a rate of 7.8 violations/1,000 herd-years (Saville *et al.*, 2000). Another retrospective study, were data included results from all

licensed dairy farms in the state of Wisconsin for the period of January 1995 through November 1998, reported a rate of detected antimicrobial residues of 4.9 violations/1000 herd years (Ruegg and Tabone, 2000). Milk quality data from March 1999 to December 2000 from five of the largest milk plants operating in New York State showed the average number of antibiotic residue violations in the pool of milk was 3.9 violations/1000 herd years. Interestingly, 82% of the residue violations were related to the treatment of mastitis in a USA study (Reneau, 1993). Also, Michigan Department of Agriculture records, collected from Michigan dairies that had an occurrence of inhibitory residues in marketed milk from 1995 to 2000, revealed that antibacterial therapy of mastitis accounts for 90% of the inhibitory residue occurrences (Erskine *et al.*, 2003). The risk of an antimicrobial residue violation was associated with the frequency of IMM antibiotic use, and so also associated with the number of clinical mastitis. Farmers with an antimicrobial residue violation reported 2.01 cows treated with antibiotics per month and controls, where as residue-free farmers reported 1.28 cows treated per month (McEwen *et al.*, 1991).

Resistance to antibiotics associated with the use of antibiotics in animals is currently an issue of principal concern. First, there are concerns about potential for transfer of antibiotic-resistant pathogens through the food chain and the risk of transfer of antibiotic-resistant genes from animal enteric flora to human pathogens. Second, there is concern of reduced efficacy of antibiotic therapy in animals colonized with resistant bacteria (Barton, 2000). Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Owens *et al.*, 1997; Erskine *et al.*, 2002; Makovec

and Ruegg, 2003; Pitkala *et al.*, 2004; Pol and Ruegg, 2007). Resistance to antimicrobial agents in mastitis pathogens has two relevant aspects: The first is a reduction in cure rates after treatment of clinical mastitis cases (Owens *et al.*, 1997; Sol *et al.*, 2000). The second issue is the potential impact of transmission of resistant bacteria to humans via the food chain (Barton, 2000). The World Health Organization (WHO) has stated that any use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacteria (WHO, 1997).

To summarize, mastitis has been recognized the most frequent reason for antibiotic use in lactating dairy cattle. Problems attributed to the use of antibiotics for the treatment of mastitis include those of antibiotic residues and concerns about development of antibiotic resistance. Given these concerns, any new techniques developed for control of mastitis must promote the judicious use of antimicrobials on farms.

ON-FARM DIAGNOSIS OF INTRAMAMMARY INFECTIONS

Microbiological Culture

Rapid on-farm culture systems may allow producers to make strategic mastitis treatment decisions, which could result in a significant reduction in on-farm antimicrobial use while maintaining or improving treatment efficacy and preserving the future production potential of the cow. Added benefits could include reduced overall treatment costs,

reduced risk for antimicrobial residues in milk, and reduced risk for the potential development of antimicrobial resistance in mastitis pathogens (Godden *et al.*, 2007).

The Minnesota Easy Culture System II (University of Minnesota, St. Paul, MN), a commercial on-farm culture system, offers two different types of selective culture media systems. The Bi-plate system is a plate with two different types of agar: MacConkey agar on one half selectively grows Gram-negative organisms, while Factor agar on the other half of the plate selectively grows Gram-positive organisms (Staphylococci and Streptococci). Alternately, the Tri-plate system is a plate with three different types of agar: in addition to including MacConkey agar (Gram-negative growth) and Factor agar (Gram-positive growth), it also includes a section of MTKT agar which is selective for Streptococci. Producers dip a sterile cotton swab into the milk sample then apply it over the media surface (estimate 0.1 ml plating volume). The plate is incubated in an on-farm incubator at 37 °C and is read at 24 hours. If no growth is observed, plates are rechecked after 48 hours then discarded.

Another commercial OFCS is the Petrifilm™ system (3M Microbiology, St. Paul, MN). Ruegg (2005) reported that Petrifilm™ products that are potentially useful for diagnosis of mastitis include Petrifilm™ Aerobic count plates, Coliform count plates and Staph Express count plates. In a recent evaluation of the Petrifilm™ method, the sensitivity and specificity of the Petrifilm™ method and standard laboratory microbiological method for isolation of *Staphylococcus aureus* was 87.5% and 65.6%, respectively (Silva *et al.*, 2005). However, the diagnosis of *Staphylococcus aureus* using the Petrifilm™ Staph

Express method was highly dependent upon the ability of the individual reading the test to observe variations in colony color.

The HyMast[®] Bacteriological Test System, a selective media bacteriological test system for detection of Gram-positive (Staphylococci, Streptococci) and Gram-negative (coliform) organisms in milk, has been shown to have high specificity and moderate sensitivity, compared with the gold standard of bacteriologic culture of milk in a laboratory setting (Jansen *et al.*, 1999). At 12 hours, the sensitivities were low and less consistent between readers ranging between 26 to 40%. With time the sensitivity improved, but still was low compared to the ability to correctly identify Gram-positive growth. At 36 hours, false-negative results varied between 33% and 56%, while false-positive results varied between 31% and 43%. The HyMast[®] test is no longer commercially available in the United States.

Somatic Cell Count

Somatic cells are comprised of leukocytes and a small percentage of epithelial cells. Leukocytes consist of macrophages, lymphocytes, and polymorphonuclear neutrophils (PMN). The macrophages are involved in immune recognition and are the predominant cell type present in milk from uninfected quarters (66-88%) (Östensson, 1988; Sandholm, 1995). Lymphocytes are responsible for immune memory. Polymorphonuclear neutrophils are the primary means of defense against an invasion of the mammary gland by microorganisms. The proportion of total somatic cell count (SCC) that is neutrophils is

only 1–11% in a healthy quarter but increases up to 90% or more in a quarter with IMI (Sandholm, 1995).

There are various automatic methods for somatic cell counting. The standard laboratory method is the electro-optical Fossomatic method (Foss, Hillerød, Denmark). Milk SCC has been used extensively as an indirect indicator of IMI. The sensitivity and specificity of using a threshold of 200,000 cells/ml for determination of quarter infection status has been evaluated in several studies. The reported sensitivities range from 73-89% and specificities from 75-85% (Ruegg and Reinemann, 2002). However, more recently it was suggested that the SCC for a composite milk sample from a cow should not exceed 100,000 cells/ml (Krömker *et al.*, 2001). Lactation stage affects the SCC; immediately after parturition SCC is high. This decreases quickly to the normal levels within 3 days in healthy quarters but remains high in the infected ones. Thus it may be possible that quarter SCC can be used early post partum to detect IMI (Barkema *et al.*, 1999). In a study of newly calved cows, when using a threshold of 100,000 cells/ml for quarter milk samples, the accuracy for detection of IMI was the best on day 5 post-calving. Using this sampling scheme the sensitivity and specificity to identify infected quarters was 57.4% and 72.3%, respectively (Sargeant *et al.*, 2001).

A recent review paper used meta-analysis to study the effect of different pathogens on quarter SCC (Djabri, 2002). The average SCC for bacteriologically negative quarters was 68,000 cells/ml, average SCC for quarters infected with minor pathogens was between 110,000 cells/ml and 150,000 cells/ml, and that for quarters infected with major

pathogens was higher than 350,000 cells/ml. The highest mean value was found in mastitis caused by coliforms and *Streptococcus uberis* (over 1 million cells/ml), and the lowest in IMI caused by corynebacteria (105,000 cells/ml).

California Mastitis Test

The California Mastitis Test (CMT) was developed in 1957 to detect abnormal milk (Schalm and Noorlander, 1957), and is an indirect measure of leukocytes in milk (Barnum and Newbould, 1961). Anionic detergents lyse white blood cells present in milk and lead to gel formation with DNA. Bromocresol purple is used as an indicator of pH. The CMT has been used since then as a rapid, inexpensive, and convenient cow-side screening tool for identification of subclinical infection in quarter milk (NMC, 1999). The CMT reagent-milk mixture gels in proportion to the number of leukocytes (somatic cells) present in the milk (CMT score system: negative, trace, 1, 2, or 3). The ability of CMT to identify quarters with IMI has been evaluated extensively, with variable results. CMT was studied as a tool to select cows for dry cow therapy, and was found to correctly identify 75–80% of the cows which needed the therapy, depending on the study and type of the mastitis pathogen (Poutrel and Rainard, 1981).

Recent studies using the CMT test in the first week after calving have suggested that there may be potential for its use as a screening tool to identify subclinical IMI in fresh cows (Sargeant *et al.*, 2001; Wallace *et al.*, 2002; Dingwell *et al.*, 2003). It was determined that using a threshold reaction of greater than zero was the optimum cutpoint

for detecting IMI. With this sample scheme, the sensitivities for detecting IMI with any pathogen, IMI with a major pathogen, and IMI with a minor pathogen were 56.7%, 66.7%, and 49.5%, respectively. Respective specificities were 56.2%, 54.8% and 56.2% (Sargeant *et al.*, 2001). In one study, where quarters were tested between 1-3 DIM and also a threshold reaction of greater than zero was considered positive, it was reported a lower sensitivity for detecting IMI with a minor pathogen and higher overall specificities (Wallace *et al.*, 2002). In this study the sensitivity and specificity of the CMT for major pathogens was 55.5% and 85.5%, respectively. For minor pathogens, the sensitivity was 18.5% and the specificity was 82%. In another study, when the test was performed in the first week after calving and any level of CMT reaction was considered to be indicative of an IMI, with a prevalence of 10% IMIs, the sensitivity, specificity, positive predictive value, and negative predictive value were 68.8%, 71.5%, 21.1%, and 95.4%, respectively (Dingwell *et al.*, 2003). Thus, if the CMT yielded a negative result, the producer could be 95% certain the quarter was truly uninfected. However, if the test yielded a positive result, there was a 79% chance that it was a false-positive result.

Newer rapid on-farm tests to measure or estimate SCC include the PortaSCC™ (PortaScience, Portland ME) and the Delaval Direct Cell Counter (DCC). In recent evaluations of these two tests, when subclinical mastitis was defined based on a threshold of 200,000 cells/ml, the agreement observed between SCC and either PortaSCC or the Delaval DCC was 88.0% and 95.4%, respectively (Ruegg, 2005).

To summarize, in order to identify quarters affected with mastitis infections that could benefit from antibiotic therapy and make judicious use of antibiotics a reality, there is a need to develop and validate tools to make a rapid and accurate on-farm diagnoses of presence and etiology of infection. Studies using the CMT test in the first week after calving have suggested that there may be potential for its use as a screening tool to identify subclinical IMI in fresh cows. However, due to the high false positive rate has been suggested that a rapid and accurate method for determining if infection is present in CMT-positive quarters, and type of pathogen present, would benefit producers. On farm culture, is a rapid and inexpensive tool, could conceivably be used to a) diagnose and guide strategic treatment of clinical mastitis cases, and b) use in conjunction as a screening tool (such as CMT), to diagnose and guide strategic treatment of subclinical IMI in cows after parturition.

LITERATURE REVIEW SUMMARY

Clinical and subclinical mastitis in dairy cattle is a common and costly disease that also has potential repercussions on public health. There is a need to develop and validate new management strategies that reduce its impact on cow health and dairy production economics, while ensuring the quality and safety dairy food products. This review discussing epidemiology and treatment of clinical mastitis during lactation, and subclinical mastitis after parturition, brings to light opportunities in treatment decisions, and introduces potentially useful new diagnostic tools.

It has been reported that more than half of cultures from clinical mastitis cases yield no growth or Gram-negative bacteria. These cases of clinical mastitis may not benefit from IMM antibiotic therapy. Conversely, Gram-positive IMI benefit from IMM therapy. The selective treatment of clinical mastitis might reduce treatment related costs, and promote judicious use of antibiotics.

Subclinical mastitis IMI at calving is highly prevalent, and has important consequences in cow health and production throughout the future lactation. The successful identification and treatment of those infections immediately after parturition before milk is saleable, has the potential to diminish the disease economical impact, while treatment related costs are reduced. However, similar to clinical mastitis, only Gram-positive IMIs may benefit from antibiotic therapy.

Finally, in order to identify quarters affected with mastitis that benefit from antibiotic therapy and make judicious use of antibiotics a reality, there is a need to develop and validate tools to make a rapid and accurate on-farm diagnoses of the presence and etiology of infection.

Qualitative measures of somatic cell count such as the CMT, though possible a useful screening tool, is unlikely to fit this purpose because of the a) high false positive rate to detect IMI, and the b) need to classify type of pathogen present.

On farm culture, a rapid and inexpensive tool, could conceivably be used to a) diagnose and guide strategic treatment of clinical mastitis cases, and b) use in conjunction as a screening tool (such as CMT), to diagnose and guide strategic treatment of subclinical IMI in cows after parturition.

The objectives of this thesis project are to:

- a) Evaluate the efficacy of an on-farm culture system for strategic treatment of clinical mastitis.
- b) Evaluate the efficacy of two programs designed to diagnose and treat subclinical intramammary infections after parturition.
- c) Validate an on-farm culture system (Bi-Plate Minnesota Easy Culture System II).

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TABLES

Table 1. 1. Incidence and etiology of clinical mastitis in the Great Lakes North-American region.

References	Incidence ¹	Etiology ² (% of samples)						
		NG	CNS	CPS	SAG	NAGS	COLIF	OTHER
Riekerink <i>et al.</i> , 2007 (Canada, Nationwide)	22.4	39.6	5.9	10.4	0.1	13.8	13.9	7.2
Sargeant <i>et al.</i> , 1998 (Canada, ON)	20.4	17.6	28.5	6.7	0.7	14.1	17.2	7.2
Erskine <i>et al.</i> , 1988 (USA, PA) - High SCC	35	8.8	---	18.3	41.5	12.6	8	---
Erskine <i>et al.</i> , 1988 (USA, PA) - Low SCC	51	28.6	---	2.2	0	12.3	43.5	---

¹Incidence = number of cases per 100 cows per year.

²NG = no growth; CNS = coagulase-negative staphylococcus; CPS = coagulase-positive staphylococcus; SAG = *Streptococcus agalactiae*; NAGS = non-agalactiae streptococcus; COLIF = coliforms; and OTHER = other pathogens.

Table 1. 2. Intramammary infection prevalence and etiology in milk samples collected within 5 days after parturition.

References	Quarter / Cow Prevalence of IMI ¹	Etiology ³ (% of samples)						
		NG	CNS	CPS	SAG	NAGS	COLIF	OTHER
Oliver <i>et al.</i> , 1997 Lact 1; 3 DIM (Trial 1)	47 (Q) ²	53	37.2	0	---	8.7	0.6	---
Oliver <i>et al.</i> , 1997 Lact 1; 3 DIM (Trail 2)	63 (Q) ²	47	53.4	1.7	---	5.0	0.8	---
Fox <i>et al.</i> , 1994 Lact 1; ≤4 DIM	36 (Q) ²	64	21.8	2.8		7.7	---	3.5
Godden <i>et al.</i> , 2003 Lact ≥2; ≤3 DIM	29 (Q) ²	71	10.5	2.6	0.12	10.2	9.4	1.1
Sargeant <i>et al.</i> , 2001 All Lact; ≤3 DIM	36 (Q) ²	64	17.9	1.9	---	5.0	1.9	3.3
Wallace <i>et al.</i> , 2004 All Lact; ≤3 DIM	31 (Q) ²	69	13.2	3.5	---	7.2	3.5	---
Roberson <i>et al.</i> , 1994 Lact 1	55 (C) ²	45	39	8	---	7	6	---
Kirk <i>et al.</i> , 1996 Lact 1	71 (C) ²	29	39	---	---	11	16	1
Roberson <i>et al.</i> , 1994 Lact ≥2	44 (C) ²	56	19	13	---	5	5	---

¹IMI = intramammary infection

²Q = quarter prevalence; C = cow prevalence.

³NG = no growth; CNS = coagulase-negative staphylococcus; CPS = coagulase-positive staphylococcus; SAG = *Streptococcus agalactiae*; NAGS = non-agalactiae streptococcus; COLIF = coliforms; and OTHER = other pathogens.

CHAPTER II

THE SELECTIVE TREATMENT OF CLINICAL MASTITIS BASED ON ON-FARM CULTURE RESULTS HALVES ANTIBIOTIC USE AND TENDS TO REDUCE MILK WITHHOLDING TIME WITHOUT AFFECTING SHORT-TERM CLINICAL AND BACTERIOLOGICAL OUTCOMES

The objective of this multi-state multi-herd clinical trial was to report on the efficacy of using an on-farm culture system to guide strategic treatment decisions in cows with clinical mastitis. Four hundred and twenty two cows affected with mild or moderate clinical mastitis in 449 quarters were randomly assigned to either a) a positive-control treatment program or b) an on-farm culture-based treatment program. Quarter cases assigned to the positive-control group received immediate on-label intramammary treatment with Cephapirin Sodium. Quarters assigned to the culture-based treatment program were not treated until the results of on-farm culture were determined after 24 hr of incubation. Quarters in the culture-based treatment program that showed Gram-positive growth or a mix infection were treated according to label instructions using intramammary Cephapirin Sodium. Quarters assigned to the culture-based treatment program that showed Gram-negative or no growth did not receive intramammary therapy. The proportion of quarters that received intramammary antibiotic therapy because of study assignment was 100% and 44% in quarter cases assigned to the positive-control

and culture-based treatment groups, respectively. The proportion of quarters that received secondary (or extended) antibiotic therapy was 36% and 19%, for cases assigned to the positive-control and to the culture-based treatment groups, respectively. There was a tendency for a reduction in days out of the tank for the milk from cows assigned to the culture-based treatment program *vs.* cows assigned to the positive-control group (5.9 *vs.* 5.2 days). There were no statistically significant differences between cases assigned to the positive-control and cases assigned to the culture-based treatment program in days to clinical cure (2.7 *vs.* 3.2 days), bacteriological cure risk within 21 days of enrollment (71 *vs.* 60%), new intramammary infection risk within 21 days of enrollment (50 *vs.* 50%) and presence of infection, clinical mastitis recurrence, or risk of removal from the herd (68 *vs.* 71%) within 21 days after the clinical mastitis case.

INTRODUCTION

Mastitis has been recognized as the most frequent reason for antibiotic use in dairy cattle (Sundlof *et al.*, 1995; Mitchell *et al.*, 1998). In a recent study in 20 Wisconsin conventional dairies, 80% of all antimicrobial drugs used were used for treatment or prevention of mastitis. Interestingly, 50% of all antimicrobial drugs used were used solely for treatment of clinical mastitis (CM) (Pol and Ruegg, 2007). Problems attributed to the use of antibiotics in animals include those of antibiotic residues and the potential development of antibiotic resistance (Owens *et al.*, 1997; Barton, 2000; Sol *et al.*, 2000; Erskine *et al.*, 2002; Makovec and Ruegg, 2003; Pitkala *et al.*, 2004; Pol and Ruegg, 2007).

Another concern, discarded milk following antibiotic treatment may account for as much as 73% of lost marketable milk, and in herds that do not have a judicious treatment program, losses from discarded milk alone can exceed \$100 per cow in the herd per year (Bartlett, 1991). In addition, economic damage due to discarded milk may be comparable with the damage caused by decreased milk production. Thus, there is increased awareness among producers of treatment-related costs and the economic costs of extensive antibacterial therapy for mastitis (Erskine *et al.*, 2003). However, contrary to the expected reduction in discarded milk in a non antibiotic treatment regimen, a clinical trial evaluating two antibiotic treatment regimens and one based just on the administration of oxytocin found that the cost of treatment, calculated by adding the cost of the therapy to the value of the milk withheld, did not differ between one of the antibiotic treatments and the non antibiotic regimen. The oxytocin treatment costs were not significantly lower than for amoxicillin because of the increased number of milkings required for some of the affected quarters of the oxytocin treated group to produce milk with a normal appearance (Van Eenennaam *et al.*, 1995).

It has been reported that 10 to 40% of cultures from CM cases yield no bacterial growth (NG) and so do not require antimicrobial therapy (Roberson *et al.*, 2003). Another 40% of positive cultures (Gram-negatives, yeast) are not susceptible to most approved intramammary (IMM) products. Also, a high proportion of Gram-negative (GN) infections are quickly cleared by the cow's own immune system (although occasional persistence of GN infections occurs) (Pyörälä *et al.*, 1994; Erskine *et al.*, 1992).

Conversely, intramammary (IMM) antibiotic therapy is routinely recommended for infections caused by Gram-positive (GP) organisms such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and environmental streptococci species. Based on these numbers, Roberson (2003) estimated that antibiotics labeled for IMM use would not be justified for 50 to 80% of CM cases.

Consequently, CM treatment decisions should be based on culture results. However, laboratory culture has not been routinely utilized by many dairies because of the time delay between submission of milk samples and reporting of results. Adoption of rapid on-farm milk culture (OFC) systems could allow producers to make strategic treatment decisions for CM cases, based on knowing the pathogen involved. The Minnesota Easy Culture System (University of Minnesota, Saint Paul, MN), a commercial OFC system, offers two different types of selective culture media systems. The Bi-plate system is a plate with two different types of agar: MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. Alternately, the Tri-plate system is a plate with three different types of agar. In addition to including MacConkey agar (GN growth) and Factor agar (GP growth), it also includes a section of MTKT agar which is selective for streptococci. The use of on-farm milk culture for the selective treatment of CM may represent a tremendous opportunity to reduce antimicrobial use on commercial dairy farms without sacrificing the efficacy of treatment or the long-term health and production potential of the cow. Benefits could include reduced economic cost of therapy, reduced risk of antimicrobial residues in milk, and a reduction in the potential risk for development of antimicrobial

resistance in mastitis pathogens. However, many of these potential benefits need further study to confirm and quantify the nature of these proposed benefits.

The objective of this study was to investigate the efficacy of using an OFC system to guide strategic treatment decisions in cows with mild and moderate CM. Outcomes evaluated included: a) risk to receive primary IMM antibiotic therapy because of study assignment, b) risk to receive secondary (or extended) IMM antibiotic therapy, c) days to return to visibly normal milk (days to clinical cure), d) days of milk withheld from market (days out of the tank), e) bacteriological cure within 21 days of enrollment, f) new intramammary infection (IMI) risk within 21 days of enrollment, and g) presence of infection, clinical mastitis recurrence, or risk of removal from herd (ICR) within 21 days of enrollment.

MATERIALS AND METHODS

Study Design

A randomized controlled field trial was conducted between June 2005 and April 2007 in 8 dairy herds. In each herd cows were enrolled into the study for a period not longer than 6 months. These herds, 2 in Minnesota, 5 in Wisconsin and 1 in Ontario, were a convenience sample of commercial dairy farms from the North American Great Lakes Region. Selected producers were required to maintain compliance with the study protocols and record keeping, have trained personnel, individual animal identification,

treatment facilities, appropriate drug storage capabilities, refrigeration and freezer capacity, participate in a Dairy Herd Improvement Association (DHIA) testing program, and demonstrate sufficient time and interest in the study. Herd size ranged from 150 to 1,800 cows, averaging 850 cows. Seven of the herds were housed in free-stalls and one in a tie-stall housing system. Annual milk production among those herds ranged from 9,500 kg to 12,800 kg, averaging 10,800 kg. Bulk tank milk SCC ranged from 180,000 cells/ml to 334,000 cells/ml, averaging 253,000 cells/ml.

Case Definition

Clinical mastitis was diagnosed if milk from one or more glands was abnormal in color, viscosity, or consistency, with or without accompanying heat, pain, redness, or swelling of the quarter, or generalized illness. All lactating cows in the herd were eligible for enrollment at the time of occurrence of CM, except cows exhibiting severe or grade 3 CM (depression, anorexia, dehydration, fever), or any cow with fewer than three functional teats.

Enrollment Process

Cows with CM were detected in the milking parlor by the milkers upon appreciation of clinical signs of mastitis (e.g. visible abnormal milk and/or quarter). If the cow met the designated inclusion criteria for enrollment, herd personnel aseptically collected a single milk sample from the affected quarter. For a first CM episode (cow not previously

enrolled into the study), eligible cows for enrollment were randomly assigned following a simple randomization schedule to either the positive-control group (PC) or culture-based treatment group (CB) by opening a pre-identified envelope following a sequential order. If more than one quarter was affected, all affected quarters were assigned to the same treatment group. For a second (or greater) CM episode in the same cow (i.e. cow had been previously enrolled), in the same or in a different quarter, the quarter was assigned to the same treatment group as was previously assigned.

Treatment Groups

Positive Control Group

Immediately after enrollment the quarter milk sample that had been collected was frozen on-farm at -20 °C and the affected quarter(s) was infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). The treatment was repeated once, 12 hours after the first treatment in according to label directions. A milk-withdrawal period of 96 hours and a slaughter withdrawal period of four days were followed after the last treatment.

Culture-Based Treatment Group

The aseptically collected milk sample(s) from the affected quarter(s) was first cultured on-farm using the Minnesota Easy Culture System (University of Minnesota, St. Paul,

MN). This OFC system consists of a bi-plate which is a petri dish with two different types of agar, MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. A sterile cotton swab was dipped into the milk sample and then plated onto the Factor media half of the bi-plate, redipped into the milk, and then applied to the MacConkey media half of the bi-plate. The plate was placed in an on-farm incubator and incubated at approximately 37°C for 24 hours. The quarter milk sample that had been collected was then frozen on-farm at -20 °C. The next day the plate was read and interpreted according to guidelines provided for the Minnesota Easy Culture System. If bacteria did not grow, the plate was returned to the incubator and re-read approximately 24 hours later. Final results for each sample plate were recorded as a) GP, when bacteria grew only in the Factor agar media of the bi-plate, b) GN, when bacteria grew only in the MacConkey agar media of the bi-plate, c) NG, when bacteria did not grow in either media, and d) mix infection when bacteria grew in both media. The decision about initiation of IMM antibiotic therapy the day after enrollment of the CM case was based on the on-farm culture results. Quarters from which GP bacteria were isolated or had a mix infection received the same IMM antibiotic treatment following the same procedures than cases assigned to PC. If the on-farm culture result was GN or NG, then the quarter did not receive IMM therapy.

After enrollment farm personnel recorded short-term outcomes including the number of days the cow was treated, the number of days to return to visibly normal milk, number of days out of tank and whether or not extended (secondary) therapy was given. Study technicians visited the study herds once per week and

aseptically collect single quarter milk samples from enrolled quarters at approximately 14 days post-enrollment (10-16 days post-enrollment) and 21 days post-enrollment (17-23 days post-enrollment). All milk samples were transported on ice to the regional mastitis culture laboratory (St. Paul, MN; Madison, WI or Guelph, ON) and frozen at -20 °C until bacteriological culture was completed.

Laboratory Bacteriological Culture

Aerobic culture methodologies for frozen milk samples (enrollment day 0, day 14, day 21) collected on farms were standardized among labs at all three participating sites and performed in accordance with the National Mastitis Council guidelines (NMC, 1999). Briefly, individual quarter milk samples were thawed at room temperature. While still cold, 0.01 ml of milk was plated onto MacConkey agar and Factor agar using sterile calibrated loops. Factor Agar, similar to KLMB agar (Beatty *et al.*, 1985), selects for GP organisms while inhibiting the growth of GN bacteria with antibiotics. Inoculated plates were incubated at 37°C. After incubation for 18 to 24 h, all plates were observed for microbial growth. Those plates having growth were recorded and species identification started. All plates were placed in the incubator for an additional 36 to 48 h and reevaluated for microbial growth. Colonies on MacConkey agar plates were presumptively identified based on colony morphology. Colony color was used as a means of determining if the organism on the plate was a lactose-fermenting organism. Isolates were also Gram stained to assist in organism identification. Organism identity was confirmed using the API 20E test (bioMerieux-Vitek, Inc. Hazelwood, MO). Colonies

suspected as being staphylococci based on morphology were confirmed as staphylococci based on catalase reaction and microscopic morphology. Organisms suspected of being *Staphylococcus aureus* were confirmed using the tube coagulase reaction. Those organisms that were catalase-positive and coagulase-negative were classified as *Staphylococcus* spp. Catalase-negative streptococci were streaked onto TKT medium, which is selective for *Streptococcus* spp. only, to determine the esculin reaction and presumptive identification prior to organism confirmation using the API Streptococcus identification system (bioMerieux-Vitek, Inc. Hazelwood, MO).

Data Analysis – Definition of Outcome Variables

Risk to Receive Primary IMM Antibiotic Therapy because of Study

Assignment

All CM cases assigned to PC were treated with two infusion syringes of (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). However, for cases assigned to CB only quarters from which GP bacteria were isolated or had a mix infection received antibiotic treatment initially.

Risk to Receive Secondary IMM Antibiotic Therapy of Non-Responsive

Cases

Secondary (or extended) treatment was allowed in cases that did not respond to the initial treatment regimen assigned. Failure to respond was defined as a) increasing severity (i.e. became a grade 3) of the CM case within 24-48 hours after the initial treatment regimen was implemented (either IMM antibiotic treatment or no treatment) or b) failure to reduce in severity (grade) of mastitis when assessed at approximately 48 hours after the initial treatment regimen was implemented.

Risk of Receiving IMM Antibiotics because of Primary or Secondary Therapy

The overall risk of receiving IMM antibiotic treatment was represented by a dichotomous outcome ($Y = 0$ or 1) denoting no administration (0) or administration (1) of IMM antibiotic therapy because either of study assignment or secondary treatment for each quarter case of mastitis.

Days to Clinical Cure

Herd personnel assessed mammary gland secretion daily after enrollment, and recorded the date and time when milk had returned to being visibly normal (no clots, no flakes).

Days Out of the Tank

The date and time when milk was first marketed after enrollment of the CM case was also recorded by herd personnel.

Bacteriological Cure Risk

A quarter was considered infected when one or two bacterial species were isolated from a quarter milk sample. The isolation of two bacterial species was considered a mixed infection. A quarter sample was considered contaminated if three or more bacterial pathogens were isolated. A bacteriological cure within a quarter was defined as the presence of one or two microorganisms in the enrollment milk sample, and the absence of the same specified microorganism(s) in both d-14 and d-21 milk samples.

New IMI Risk

A quarter was considered newly infected whenever a new bacterial species that was not previously present in the enrollment sample (d-0) was isolated from quarter milk samples collected either at d-14 or d-21 after enrollment.

ICR Risk

The presence of infection, clinical recurrence, or removal from herd because of culling or death was described as a dichotomous outcome ($Y = 0$ or 1) called ICR risk. Presence of infection ($I = \text{infection}$) represented the absence (0) or presence (1) of infection in quarter

milk samples collected either at d-14 or d-21 samples after enrollment. Quarters cases that could not be resampled at d-14 or d-21 after enrollment because the quarters experienced a recurrent case of CM (C = Clinical) during the follow-up period were assigned an ICR risk value of 1. Similarly, quarter cases that could not be resampled at d-14 or d-21 because cows were removed from the herd because of culling or death (R = Removed) were assigned an ICR risk value of 1. Analysis of the parameter called ICR risk was done in an attempt to eliminate potential omission bias created by not including, in the bacteriological cure analysis, cases where no bacteria were isolated from the enrollment sample, and cases without a follow-up culture result because of CM recurrence or because the cow was removed from the herd.

Statistical Analysis - Models and Modeling Strategy

Database summaries and plots were used for exploratory data analysis. Basic diagnostics techniques were used to evaluate normality, independence, homoscedasticity, collinearity and linearity of variables.

Generalized Linear Mixed Models for Dichotomous Outcome Variables

Binary response variables such as risk of being assigned to receive primary IMM antibiotic therapy, risk for secondary IMM antibiotic therapy, risk of receiving IMM antibiotic therapy because of study assignment or secondary treatment, quarter risk for a bacteriological cure, quarter I risk, quarter ICR risk and quarter risk for a new IMI, were

modeled as a function of treatment group and other covariates using logistic multivariable regression. The treatment effect on the risk for the listed outcome variables following the CM case was analyzed by generalized linear mixed models using the GLIMMIX PROC of SAS version 9.1 (SAS Institute, Cary, NC) with cow and herd included as random effects.

Covariates such as cow parity, days in milk (DIM) at CM event, previous occurrence of a CM case in the same quarter in the present lactation, number of quarters affected, and etiology of infection were included in the model if it was a potential confounding variable. To determine if a covariate confounded the treatment effect on the outcome, the crude estimate of treatment group (PC vs. CB) was compared with the adjusted estimate for that third confounding variable. It was concluded that the variable confounded the association between treatment group and outcome variable if the ratio between the difference of the crude estimate and the adjusted estimate versus the crude estimate was greater than 10%. Each variable was examined for potential confounding one at a time by regression. Once the confounder variables were identified, the next step was to place all confounders into a full model with two-way interaction terms between treatment and the confounder. In order to simplify the model each non-significant interaction term was removed one at a time using a backward stepwise approach, starting by the least significant interaction term, and running the model again until there were no non-significant interaction terms in the model. Next, with non-significant interaction terms removed from the model, it was determined whether there were main effect variables in the model that were not in an interaction term that might be a confounder. The least

significant term were removed and it was evaluated if this affected the treatment effect estimate, with the goal to assess whether the variable confounded the treatment-outcome relationship. If the variable was an important confounder, it was returned to the model and other variables were assessed one at a time to see if they were confounders. The treatment variable was forced in the model regardless of the P -value. Once all non-significant interaction terms were removed as well as main effect variables that did not confound the exposure-outcome relationship, this was the final model. Final significance was declared at $P < 0.05$.

Time to Event Models

Binary responses with a “time to event” component such as days to clinical cure and days out of the tank were modeled using survival analysis. Cox’s proportional hazards regression method was used to test the logistic analysis explanatory variables (see previously described covariates) simultaneously for their association with time until event (PROC PHREG). The standard model was extended by including a frailty term reflecting a latent effect associated with each herd and with each cow when the event of interest was at the quarter level.

Cows (and quarters) were censored when the event of interest happened or when further follow-up data was not available for the days to return to visibly normal milk and the days of milk withheld from market analysis. The assumption of independent censoring between both treatment groups was assessed by comparing the proportion of censored

cows or quarters between both treatment groups. In addition, a sensitivity analysis looking at situations of complete positive correlation (every cow or quarter censored experienced the event of interest) or negative correlation (censored cows or quarters did not experienced the event of interest) between censoring and the event of interest was done. If the violation of this assumption did not dramatically alter the treatment effect estimate (<10%), it was concluded that censoring did not introduce bias.

RESULTS

Descriptive Data

Four hundred and twenty two cows affected with CM in 449 quarters were enrolled in the study. Two hundred and fourteen cows with 229 affected quarters were assigned to PC, and 208 cows with 220 affected quarters were assigned to CB. Cow and quarter level descriptors and etiology of infection at enrollment for both study groups are shown in Table 2.1. The severity distribution of the clinical cases enrolled in the study was 68% for mild cases and 32% for moderate cases. Severe cases of mastitis were not eligible for enrollment in this study. The parity distribution of the cows at the time of the CM event was 33%, 30% and 37% for first, second and third or greater parity cows, respectively. The mean and median DIM to the occurrence of a CM case was 170 and 150 days, respectively. Cows could have one or more quarters affected with CM when enrolled in the study. Ninety percent of the cows had just one quarter affected, and the remainder 10% had 2 or more quarters affected.

Bacteria were isolated from 66% of quarters with CM at enrollment. Coliform bacteria were the most commonly isolated pathogen (24% of CM cases), followed by non-agalactiae streptococci (14% of CM cases), coagulase-negative staphylococci (7% of CM cases), *Staphylococcus aureus* (7% of CM cases), and other infections (7% of CM cases). Among coliforms, bacteria such as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. represented 18%, 5% and 1% of all cases, respectively. Other GN such as *Pantoea* spp., *Pseudomonas* spp. and *Salmonella* spp. each represented less than 1% of all cases. *Streptococcus agalactiae* was not isolated from any of the CM cases. The non-agalactiae streptococci *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Enterococcus* spp. and *Aerococcus* spp. represented 5%, 2%, 4% and 1% of all cases. It is remarkable that *Bacillus* spp. was isolated in 4% of the CM cases. Other bacteria such as *Corynebacterium bovis* and *Arcanobacterium pyogenes* each represented less than 1% of all cases.

CM Treatment Programs Effects

Risk to Receive Primary IMM Antibiotic Therapy because of Study

Assignment

The risk for a quarter case to receive IMM antibiotic therapy as a consequence of the CB program treatment decision was reduced to 44%. It ranged from 31% to 89% for the 8 dairy herds enrolled in the study. The etiologic classification distribution based in OFC

for cases assigned to CB that did not receive IMM antibiotic therapy was 27% NG and 30% GN, and for cases that did receive IMM antibiotic was 39% GP and 4% mix infections.

Risk to Receive Secondary IMM Antibiotic Therapy of Non-Responsive Cases

The risk for a quarter case to receive secondary (or extended) IMM antibiotic therapy was lower for cases assigned to CB than for cases assigned to PC [OR_{PC} (95% CI) = 0.4 (0.3, 0.7); P = 0.0018] (Table 2.2). This risk was numerically lower in cases assigned to CB in 6 of the 8 herds enrolled in the study. No other covariates in addition to the explanatory variable of interest, treatment program, remained in the model because of confounding the treatment program effect on the risk to receive secondary IMM antibiotic therapy.

Secondary IMM antibiotic therapy was administered in 36% of the cases assigned to PC and in 19% of the cases assigned to CB. The overall risk to receive secondary IMM antibiotic therapy for both CM treatment programs was higher in GP or GN cases, 30% and 42% respectively, than in NG cases, 11%. The risk to receive secondary IMM antibiotic therapy was higher for CP for the previously mentioned CM etiology classification groups. However, although the treatment effect was not different among the three CM etiology classification groups (P = 0.2815), the relative risk for secondary IMM antibiotic therapy for cases assigned to PC and CB was numerically greater for GN cases, 16% vs. 7%, or NG cases, 57% vs. 23%, than in GP cases, 34% vs. 26%, respectively. In

cases assigned to CB, secondary therapy was administered in 28% of cases that received IMM antibiotic therapy as part of the study treatment decision at enrollment, and in 13% of cases that did not receive IMM antibiotic therapy at enrollment.

Risk of Receiving IMM Antibiotics because of Primary or Secondary Therapy

The risk for a quarter case to receive IMM antibiotic therapy because either study assignment or secondary treatment was half for cases assigned to CB than for cases assigned to PC [OR_{PC} (95% CI) = 0.009 (0.002, 0.04); $P = <0.0001$] (Table 2.2). Fifty one of the cases assigned to CB received antibiotic treatment (44% because of study assignment and 7% because of secondary treatment of cases not treated initially with antibiotics).

Days to Clinical Cure

There was no significant difference in days to return to visible normal milk between treatment programs [HR_{PC} (95% CI) = 0.8 (0.6, 1.2); $P = 0.2581$] (Table 2.2 and Figure 2.1). This time was numerically shorter in cases assigned to PC in 5 of the 8 herds enrolled in the study. The only covariate that remained in the model because of confounding the treatment program effect on days to return to visible normal milk was severity of the CM case.

The mean days for milk to return to being visible normal was 2.7 days for cases assigned to PC and 3.2 days for cases assigned to CB. The overall days out of the tank for both CM treatment programs for NG, GN and GP cases were 2.8 days, 3.2 days and 3.1 days, respectively.

Days out of the Tank

There was a tendency for lower days of milk withheld from the market for cases assigned to CB than for cases assigned to PC [HR_{PC} (95% CI) = 1.2 (0.9, 1.4); $P = 0.0797$] (Table 2.2 and Figure 2.2). This time was numerically shorter in cases assigned to CB in 5 of the 8 herds enrolled in the study. Covariates that remained in the model because of confounding the treatment program effect on days of milk withheld from the market were number of quarters affected and etiology of infection.

The average days of milk withheld from the market was 5.9 days for cases assigned to PC and of 5.2 days of for cases assigned to CB. The difference in days of milk withheld from the market between PC and CB was much greater for GN cases, 6.2 vs. 4.9 days, or NG cases, 5.5 vs. 3.9 days, than in GP cases, 6.1 vs. 6.5 days.

Quarter Milk Bacteriological Culture Follow-Up

Bacteriological Cure Risk

There was no significant difference in risk for a bacteriological cure between the two treatment programs [OR_{PC} (95% CI) = 0.6 (0.3, 1.4); P = 0.2034] (Table 2.3). The only covariate that remained in the model because of confounding the treatment program effect on bacteriological cure risk was etiology of infection.

The proportion of quarters with bacteriological cure was 71% and 60% for cases assigned to PC and to CB, respectively. The overall bacteriological cure risk for both CM treatment programs for GN and GP cases was 78% and 55%, respectively.

New IMI Risk

There was no significant difference in risk for new IMI between treatment programs [OR_{PC} (95% CI) = 1.0 (0.6, 1.6); P = 0.9416] (Table 2.3). Covariates that remained in the model because of confounding the treatment program effect on new IMI risk included number of quarters affected and etiology of infection.

The proportion of quarters with a new IMI was 50% for cases assigned to both CM treatment programs. The overall risk for new IMIs for both CM treatment programs for NG, GN and GP cases was 52%, 51% and 55%, respectively.

ICR Risk

There were no significant differences in the ICR risk (represents the presence of infection risk, CM recurrence risk, or removal from herd (ICR) risk between both treatment programs [OR_{PC} (95% CI) = 1.1 (0.7, 1.8); P = 0.7254] (Table 2.3). Covariates that remained in the model because of confounding the treatment program effect on the ICR risk included number of quarters affected and etiology of infection.

The ICR risk was 68% and 71% for cases assigned to PC and to CB, respectively. The partial contribution to the overall 69% ICR risk for both CM treatment programs was 64% for the infection risk at 14 or 21-d after enrollment, 4% for the CM recurrence risk, and 1% for the culling or death risk during this time. The ICR risk for NG, GN and GP cases was 59%, 72% and 80%, respectively.

DISCUSSION

In this study bacteria culture results NG and GN represented 36% and 26% of all CM cases, respectively. It has been previously reported that there is a shift towards environmental pathogens as the major causes of CM in the USA, Canada and several European Countries (Anon, 2001; Green and Bradley, 1998). In the North American Great Lakes region coliforms were recognized as a major etiology of CM 20 and 10 years ago (Sargeant *et al.*, 1998; Erskine *et al.*, 1988). However, recently NG cases are the most prevalent as was found in this and other studies (Wilson *et al.*, 2004; Riekerink *et al.*, 2007).

Roberson (2003) estimated that antibiotics labeled for IMM use would not be justified for 50 to 80% of CM cases. This estimate was based on the assumption that cases where bacteria were not isolated or coliform infections did not benefit from IMM antibiotic therapy. In this study NG and GN cases accounted for 60% of all cases. The use of the Bi-Plate Minnesota Easy Culture System allowed herd personnel to identify these cases and make a cow-side treatment decision the day after detection of the CM case. As a result, only 44% of the cases assigned to CB received IMM antibiotic therapy as the initial treatment decision. However, because secondary treatment decisions, the final IMM antibiotic treatment risk for the CB program was 51%.

The higher secondary treatment risk experienced by cases assigned to PC, where all CM cases were treated initially with antibiotics, may be explained in part by the possibility that herd personnel were more prone to continue antibiotic treatment, once already started, than for cases assigned to CB where more than half of the cases were not treated initially. Thus, in cases assigned to CB, secondary therapy was administered in 28% of cases that received IMM antibiotic therapy as part of the study treatment decision at enrollment, these being the cases identified on-farm as GP or mixed infections. Similarly, secondary treatment was administered in 34% of cases assigned to PC where GP bacteria was isolated. Conversely, in cases assigned to CB, secondary therapy was administered in only 13% of cases that did not receive IMM antibiotic therapy at enrollment, these being the cases identified as NG or GN by OFC. The secondary treatment risk for NG and GN cases assigned to PC was 35%. Other explanations for the higher secondary treatment risk experienced by cases assigned to PC could be the risk for contamination when

infusing the product via the teat canal (Erskine, 2003), and possible irritation of the mammary tissue caused by the preparation. In addition, some earlier in vitro studies showed that antimicrobials may disturb phagocytosis when given IMM (Nickerson *et al.*, 1986; Ziv *et al.*, 1983), but clinical relevance of this finding is unknown.

Problems attributed to the use of antibiotics in food producing animals include those of increased risk of antibiotic residue violations and the potential for development of antibiotic resistance. Different reports found that the majority of residue violations are related to the antibacterial therapy of mastitis, accounting for 82% and 90% of the inhibitory residue occurrences, respectively (Reneau, 1993; Erskine *et al.*, 2003).

Furthermore, it has been established that the risk of an antimicrobial residue violation is associated with the frequency of IMM antibiotic use on farm, and so also associated with the number of clinical mastitis cases treated with antibiotics. Farmers with an antimicrobial residue violation reported 2.01 cows treated with antibiotics per month, where as residue-free farmers reported 1.28 cows treated per month (McEwen *et al.*, 1991). The public health impact of reducing antibiotic use for the treatment of clinical mastitis, due to risk avoidance, may far outweigh the on-farm cost savings derived from a reduction in antibiotic use.

Resistance to antibiotics associated with the use of antibiotics in animals is currently an issue of principal concern. The Center for Veterinary Medicine of the Food and Drug Administration (FDA), in cooperation with the American Veterinary Medical Association (AVMA), compiled fifteen general principles for the judicious use of antimicrobials for

dairy cattle veterinarians (FDA–Center for Veterinary Medicine, 2008). These principles emphasize the use culture to aid in the selection of antimicrobials, confine the use of antimicrobials to appropriate clinical indications and limit therapeutic antimicrobial treatment to the fewest animals indicated. The selective treatment of CM based in OFC results implements those principles by reducing antibiotic use by half without a reduction in treatment efficacy. If 50% of all antimicrobial drugs used in dairy farms are dedicated to CM treatment (Pol and Ruegg, 2007), the selective treatment of CM based in OFC results has the potential to reduce total antibiotic use on dairy farms by 25%.

Discarded milk following treatment may account for as much as 73% of lost marketable milk, and in herds that do not have a judicious treatment program, losses from discarded milk alone can exceed \$100 per cow in the herd per year (Bartlett, 1991). In the present study, there was a tendency for lower days of milk withheld from the market for cases assigned to CB than for cases assigned to PC. Milk from all cows assigned to PC required at least 96 hours milk withdraw period because of IMM antibiotic treatment while only 44% of the cows assigned to CB were treated with antibiotics. Conversely, treatment was delayed one day in cases assigned to CB. The end result was a tendency for almost one day reduction in days out of the tank for the milk from cows assigned to CB. The expected reduction in discarded milk in a non-antibiotic treatment regimen has not always been reported in previous studies. A clinical trial evaluating two antibiotic treatment regimens and one based just in the administration of oxytocin found that the cost of treatment, calculated by adding the cost of the therapy to the value of the milk withheld, did not differ between one of the antibiotic treatments and the non-antibiotic

regimen (Van Eenennam et al., 1995). However, authors recognized that the milk withhold costs for the cows in the oxytocin group included milkings during which cows were producing grossly normal milk following their recovery from mastitis, but those cows still remained in the hospital string to allow sample collection along with their contemporary antibiotic treatment group.

There were no significant differences in days to clinical cure between both CM treatment programs. Results from previous studies are contradictory. In a clinical trial when comparing clinical cure risk between antimicrobial and no antimicrobial CM treatments, days to clinical cure did not differ for mild CM cases where no bacteria, streptococci or coliforms were isolated (Guterbock *et al.*, 1993). Similarly, a study where cows were experimentally infected with *Escherichia coli* and developed moderate and severe CM found no differences in days to clinical cure between antimicrobial and no antimicrobial CM treatments (Leininger *et al.*, 2003). Conversely, another study reported that when CM was caused by *Streptococcus* spp. or coliform bacteria, the clinical cure risk by the tenth milking was significantly greater if antibiotics were used (Morin *et al.*, 1998). The present study compares two different CM treatment programs, not just antimicrobial and no antimicrobial treatment of CM. Nevertheless, the clinical cure risk was not different for NG or GN cases when they were assigned to PC that includes antibiotic treatment of those cases assigned to CB that does not includes antibiotic treatment. Duration of clinical signs in the present study (mean, 3.0 days) was shorter than that reported in previous studies (5.4 days and 4.1 days) (Constable *et al.*, 2002; Hoe *et al.*, 2005). This difference may be attributable to the omission of severe cases of mastitis in the present

study, although differences in clinical cure or CM case definition, cow and herd factors may also explain the differences in duration of the clinical signs.

The bacteriological cure risk was not different between CM treatment programs. Cases where coliforms were isolated had the highest bacteriological cure, closely followed by *Bacillus* spp., similar bacteriological cure risk for cases where non-agalactiae streptococci and coagulase-negative staphylococci were isolated, and the lowest bacteriological cure was observed for *Staphylococcus aureus* cases. There was a large numerical difference in the risk of a bacteriological cure between both CM treatment programs for cases where *Staphylococcus aureus* was isolated, 43% vs. 18%. This difference could be due to chance, to the one day delay in initiating IMM antibiotic treatment, or due to a failure of the on-farm culture to identify these infections. The latter reason can be discounted as all cases assigned to CB where *Staphylococcus aureus* was isolated in the laboratory were identified as GP and treated with antibiotics on-farm. It is not possible to discern between the two first hypotheses provided. However, given the fact that these cure risks are based on only 7 and 11 cases of CM respectively, it is very possible that these cure risk differences may be numerically different due to chance alone, and so these results should be interpreted with caution.

The ICR risk and etiology of infection at 14 and 21-d after enrollment did not differ between treatment groups either numerically nor statistically. The ICR risk may be a better outcome measure of both programs treatment decisions than bacteriological cure risk since it represents both the bacteriological cure risk and new infection risk

immediately after the CM event, includes in the analysis those CM cases where bacteria were not isolated from the enrollment sample (40% of the cases). Thus, it may more truly reflect the success of the intervention in the quarter infection status. The inclusion, in the calculation of the ICR risk, of cases without a follow-up culture result because of CM recurrence or removal from the herd was also done in an attempt to reduce potential omission bias. Using the ICR risk as a dependent variable, there were no differences between both CM treatment groups for those outcomes from 14 to 21-d after enrollment.

The omission of CM cases where bacteria were not isolated from the enrollment sample (40% of the cases in the present study) in the bacteriological analysis could introduce selection bias due to omission of data from the analysis. In a clinical trial where treatment groups were balanced by randomization in the etiologic distribution of CM, the quarter bacteriological status treatment effect still could be biased if the rate of new infections for cases where bacteria were not isolated differs between treatment groups. This could conceivably happen if one of the treatment programs had a protective effect or else induced new infections in cases where bacteria were not isolated in the enrollment sample. It is our recommendation that future studies evaluating the efficacy of mastitis treatment programs should evaluate the treatment effect in quarter infection status during the follow-up milk sampling on cases where bacteria is not isolated. If instead of evaluating the efficacy of a treatment program, the objective of the clinical trial is to evaluate the efficacy of an antimicrobial drug for the treatment of clinical mastitis, then the interest of the effect of treatment on bacteriological cure may be restricted to cases where bacteria are isolated before the application of antibiotic treatment (Schukken *et al.*, 1995).

Selection bias in cases without a follow-up culture result because of CM recurrence or the cow was removed from the herd may occur if there is a differential loss to follow-up between treatment groups. Those losses could be due to differential management of cases assigned to the different groups or truly due to a treatment effect.

This study compared two CM treatment programs that differed not just on the selective vs. blanket IMM antibiotic treatment of CM. The success of the CB program also depends on the accuracy of the OFC system and the effects of a one day delay to initiate IMM antibiotic therapy in those quarters selected for treatment. Previous field trials and trials with experimentally induced coliform mastitis have already reported on the inefficacy of antimicrobial treatment (Guterbock *et al.*, 1993; Roberson *et al.*, 2004; Hallberg *et al.*, 1994; Van Eenennaam *et al.*, 1995; Leininger *et al.*, 2003). In addition, it had been reported that treatment of mild or moderate CM cases can be postponed for 1 day with minimal adverse effects while producers wait for OFC results (Wagner *et al.*, 2007). The present study is the first to evaluate a CM selective treatment decision based on OFC, made the next day after the CM event. Therefore, strengths of this study are related to the validation of a program to treat clinical mastitis.

One potential limitation of this study is that the label of IMM antibiotic administered in this study, Cefa-Lak[®] (Fort Dodge Animal Health, Fort Dodge, IA) does not include efficacy claims against Gram-negative bacteria. Two IMM antibiotic preparations, Hetacin-K[®] (Fort Dodge Animal Health, Fort Dodge, IA) and Spectramast[®] (Pfizer Animal Health, New York, NY), are currently approved in the USA with a label that

claims efficacy against clinical mastitis in lactating dairy cattle associated with *Escherichia coli*. However, published peer reviewed studies are lacking that reporting the efficacy of these two antimicrobial formulations to treat clinical mastitis cases where *Escherichia coli* or other GN pathogens are isolated. There is a need for controlled field trials evaluating their efficacy in treating clinical mastitis. Furthermore, clinical mastitis selective treatment programs based in OFC culture results using these IMM antibiotic preparations should be evaluated. Until this scientific knowledge becomes available, the validity of the present study results when using antibiotics other than cephalosporin sodium is not known.

CONCLUSIONS

The use of an OFC system to guide the strategic treatment of CM reduced IMM antibiotic use by half and tended to reduce withholding time by one day, without significant differences in days to clinical cure, bacteriological cure risk, new infection risk and ICR risk within 21 days after the CM event. Results of this study, in addition to long-term outcomes (to be reported separately), will be used to evaluate the overall cost-benefit of using an on-farm culture system to guide strategic treatment decisions in cows with mild and moderate CM.

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TABLES

Table 2. 1. Cow and quarter level clinical mastitis cases descriptors and etiology of infection at enrollment for both study groups.

Parameter	Positive-Control		Culture-Based	
	Cows ^a	Herds ^b	Cows	Herds
Sample Size				
Number of Quarters Enrolled	229	---	220	---
Number of Cows Enrolled	214	---	208	---
Severity – Quarter Level				
Mild (1 st grade)	63 (145)	10-95	72 (159)	20-100
Moderate (2 nd grade)	37 (84)	5-90	28 (61)	0-80
Parity – Cow Level				
1 st	34 (78)	0-57	32 (70)	10-57
2 nd	33 (76)	17-50	26 (56)	22-50
3 rd +	33 (74)	13-83	42 (93)	14-75
DIM – Cow Level				
Mean	174 (228)	125-224	166 (219)	119-195
Median	155 (228)	117-221	143 (219)	56-238
Quarters Affected – Cow Level				
1	88 (201)	75-100	93 (205)	80-100
2+	12 (28)	0-25	7 (15)	0-20
Previous Clinical Mastitis Event in Current Lactation – Cow Level				
No	78 (178)	67-88	75 (165)	61-100
Yes	22 (51)	12-33	25 (55)	0-39
Etiology – Quarter Level				
No growth	33 (71)	23-42	35 (74)	10-52
Gram-negatives	28 (60)	0-44	24 (50)	0-80
<i>Escherichia coli</i>	21 (44)	0-67	16 (34)	0-41
<i>Klebsiella</i> spp.	5 (10)	0-10	6 (13)	0-66
Gram-positives	33 (69)	11-63	38 (80)	10-63
Non-ag. streptococci	12 (25)	6-24	15 (32)	9-38
<i>Staphylococcus</i> spp.	8 (16)	0-13	10 (22)	0-14
<i>Staph. aureus</i>	6 (13)	0-31	8 (17)	0-38
<i>Bacillus</i> spp.	5 (11)	0-27	3 (6)	0-20
Other	10 (21)	0-20	4 (8)	0-13
Mix Infection / Contaminated	<1 (1)	0-22	2 (4)	0-15

^a Cow and quarter level descriptors [% (n)]

^b Herds range for the different descriptors [min herd (%) – max herd(%)]

Table 2. 2. Risk to receive primary IMM antibiotic therapy, risk to receive secondary IMM antibiotic therapy, risk to receive primary or secondary IMM antibiotic therapy, days to clinical cure and days out of the tank two clinical mastitis treatment programs (short-term outcomes).

Parameter	Positive-Control		Culture-Based		Treatment Effect	
	Cows ^a	Herds ^b	Cows	Herds	Estimate (95% CI)	P-value
Risk to Receive Primary IMM Antibiotic Therapy^c [% (n)]						
Quarter Level	100 (229)	100	44 (220)	31-89		
Cow Level	100 (214)	100	44 (208)	31-89		
Risk to Receive Secondary IMM Antibiotic Therapy^d [% (n)] – Quarter Level						
No growth	16 (69)		7 (73)			
Gram-negatives	57 (60)		23 (48)			
Gram-positives	34 (67)		26 (80)			
All cases	36 (224)	14-70	19 (217)	8-56	OR _{PC} = 0.4 (0.3, 0.7)	0.0018
Risk to Receive IMM Antibiotic Therapy^e [% (n)]						
Quarter Level	100 (229)	100	51 (220)	36-100	OR _{PC} = 0.009 (0.002, 0.04)	<0.0001
Cow Level	100 (214)	100	51 (208)	36-100	OR _{PC} = 0.009 (0.002, 0.04)	<0.0001
Days to Clinical Cure [Mean ± SD (n)] – Cow Level						
No growth	2.7 ± 1.3 (61)		3.0 ± 1.7 (51)			
Gram-negatives	3.1 ± 2.0 (57)		3.4 ± 1.5 (39)			
Gram-positives	2.6 ± 1.1 (54)		3.5 ± 1.6 (62)			
All cases	2.7 ± 1.5 (196)	2.2-3.5	3.2 ± 1.7 (163)	2.6-3.8	HR _{PC} = 0.8 (0.6, 1.2)	0.2581
Days Out of the Tank [Mean ± SD (n)] – Cow Level						
No growth	5.5 ± 2.6 (63)		3.9 ± 3.1 (58)			
Gram-negatives	6.2 ± 2.5 (58)		4.9 ± 2.7 (41)			
Gram-positives	6.1 ± 3.6 (62)		6.5 ± 3.7 (72)			
All cases	5.9 ± 2.9 (183)	5.0-6.3	5.2 ± 3.5 (184)	3.6-8.3	HR _{PC} = 1.2 (1.0, 1.4)	0.0797

^a Cow and quarter level descriptors

^b Herds range for the different descriptors [min herd (%) – max herd(%)]

^c Risk to receive IMM antibiotic therapy because of study assignment (primary)

^d Risk to receive IMM antibiotic therapy because of a severity assessment decision after enrollment (secondary)

^e Risk to receive either primary or secondary IMM antibiotic therapy

Table 2. 3. Quarter level bacteriological cure risk, new IMI risk, I risk, and ICR risk at 14±3 and 21±3 days after enrollment for two clinical mastitis treatment programs (bacteriology outcomes).

Parameter	Positive-Control		Culture-Based		Treatment Effect	
	Cows ^a	Herds ^b	Cows	Herds	Estimate (95% CI)	P-value
Bacteriological Cure Risk [% (n)] – Quarter Level						
Gram-negatives	86 (42)		70 (37)			
<i>Escherichia coli</i>	83 (30)		78 (22)			
<i>Klebsiella</i> spp.	86 (7)		62 (13)			
Gram-positives	59 (46)		52 (48)			
Non-ag streptococci	57 (14)		61 (18)			
<i>Staphylococcus</i> spp.	53 (15)		54 (13)			
<i>Staph. aureus</i>	43 (7)		18 (11)			
<i>Bacillus</i> spp.	71 (7)		75 (4)			
All cases	71 (97)	42-100	60 (85)	24-100	OR _{PC} = 0.6 (0.3, 1.4)	0.2034
New IMI Risk [% (n)] – Quarter Level						
No growth	50 (54)		53 (62)			
Gram-negatives	52 (44)		49 (41)			
<i>Escherichia coli</i>	43 (30)		41 (22)			
<i>Klebsiella</i> spp.	43 (7)		55 (11)			
Gram-positives	54 (54)		55 (56)			
Non-ag streptococci	53 (19)		63 (24)			
<i>Staphylococcus</i> spp.	55 (11)		40 (10)			
<i>Staph. aureus</i>	14 (7)		22 (9)			
<i>Bacillus</i> spp.	50 (6)		33 (3)			
All cases	50 (163)	25-75	50 (160)	20-63	OR _{PC} = 1.0 (0.6, 1.6)	0.9416
I Risk [% (n)] – Quarter Level						
No growth	50 (54)		53 (62)			
Gram-negatives	63 (43)		68 (41)			
<i>Escherichia coli</i>	58 (26)		57 (30)			
<i>Klebsiella</i> spp.	85 (13)		63 (8)			
Gram-positives	77 (56)		79 (62)			
Non-ag streptococci	80 (25)		79 (19)			
<i>Staphylococcus</i> spp.	67 (12)		73 (11)			
<i>Staph. aureus</i>	82 (11)		78 (9)			
<i>Bacillus</i> spp.	80 (5)		75 (8)			
All cases	62 (165)	25-83	65 (168)	25-74	OR _{PC} = 1.1 (0.7, 1.8)	0.7675
ICR Risk [% (n)] – Quarter Level						
No growth	55 (58)		62 (65)			
Gram-negatives	71 (48)		73 (41)			
<i>Escherichia coli</i>	68 (34)		65 (26)			
<i>Klebsiella</i> spp.	67 (9)		85 (13)			
Gram-positives	79 (58)		81 (64)			
Non-ag streptococci	80 (20)		80 (25)			
<i>Staphylococcus</i> spp.	73 (11)		79 (14)			
<i>Staph. aureus</i>	78 (9)		82 (11)			
<i>Bacillus</i> spp.	88 (8)		80 (5)			
All cases	68 (181)	33-83	71 (174)	25-80	OR _{PC} = 1.1 (0.7, 1.8)	0.7254

^a Cow and quarter level descriptors [% (n)]

^b Herds range for the different descriptors [min herd (%) – max herd(%)]

Figure 2. 1. Kaplan-Meier survival graph representing the probability of a clinical cure at a given days after the clinical mastitis event for two clinical mastitis treatment programs. Clinical mastitis cases assigned to the positive-control treatment program are represented by a solid line and cases assigned to the culture-based treatment program are represented by a dashed line.

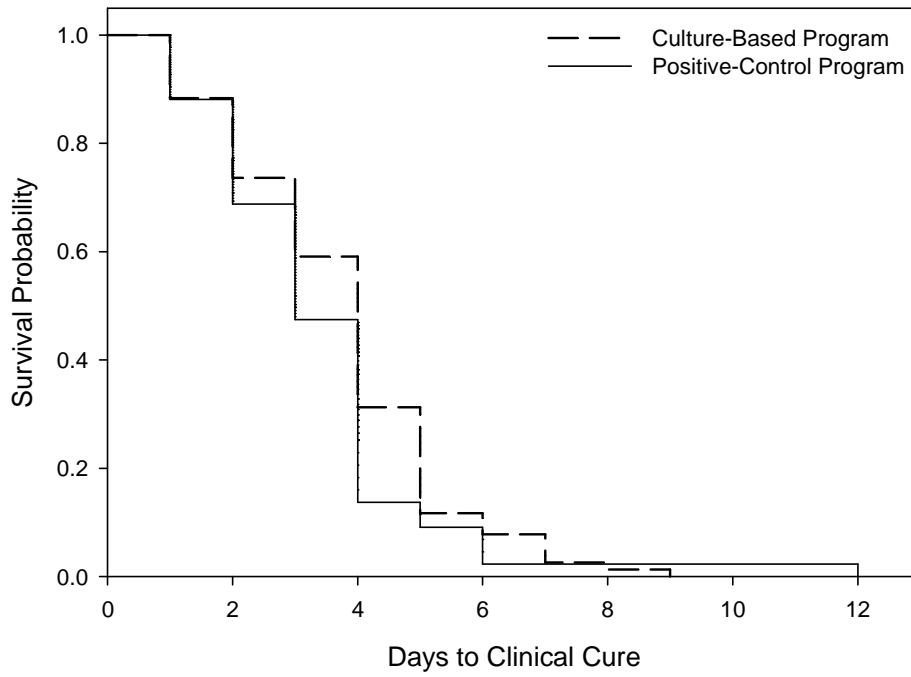
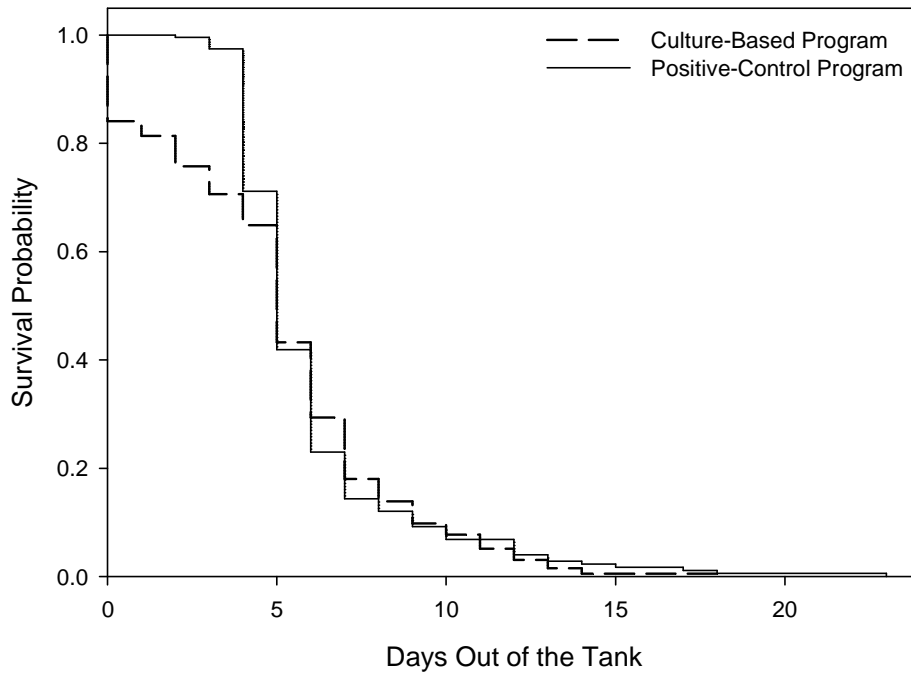


Figure 2. 2. Kaplan-Meier survival graph representing the probability of milk to return to tank at a given days after the clinical mastitis event for two clinical mastitis treatment programs. Clinical mastitis cases assigned to the positive-control treatment program are represented by a solid line and cases assigned to the culture-based treatment program are represented by a dashed line.



CHAPTER III

THE SELECTIVE TREATMENT OF CLINICAL MASTITIS BASED ON ON-FARM CULTURE RESULTS DOES NOT AFFECT LONG-TERM OUTCOMES: CLINICAL MASTITIS RECURRENCE, SOMATIC CELL COUNT, MILK PRODUCTION AND COW SURVIVAL

The objective of this multi-state multi-herd clinical trial was to report on the efficacy of using an on-farm culture system to guide strategic treatment decisions in cows with clinical mastitis. Four hundred and twenty two cows affected with mild or moderate clinical mastitis in 449 quarters were randomly assigned to either a) a positive-control treatment program or b) an on-farm culture-based treatment program. Quarter cases assigned to the positive-control group received immediate on-label intramammary treatment with Cephapirin Sodium. Quarters assigned to the culture-based treatment program were not treated until the results of on-farm culture were determined after 24 hr of incubation. Quarters in the culture-based treatment program that showed Gram-positive growth or a mix infection were treated according to label instruction using intramammary Cephapirin Sodium. Quarters assigned to the culture-based treatment program that showed Gram-negative or no growth did not receive intramammary therapy. It was already reported elsewhere that the selective treatment of clinical mastitis based in on-farm culture results reduces antibiotic use by half and tends to reduce milk

withholding time without affecting short-term clinical and bacteriological cure outcomes (Lago *et al.*, 2009). The present article reports on long-term outcomes of the mentioned study. There were no statistically significant differences between cases assigned to the positive-control and cases assigned to the culture-based treatment program in risk and days for recurrence of clinical mastitis in the same quarter (35% and 78 days *vs.* 43% and 82 days), linear somatic cell count (4.2 *vs.* 4.4), daily milk production (30.0 *vs.* 30.7 kg), and risk and days for culling or death events (28% and 160 days *vs.* 32% and 137 days) for the rest of the lactation after enrollment of the clinical mastitis case.

INTRODUCTION

Mastitis has been recognized as the most frequent reason for antibiotic use in dairy cattle (Sundlof *et al.*, 1995; Mitchell *et al.*, 1998; Pol and Ruegg, 2007). Problems attributed to the use of antibiotics in animals include those of antibiotic residues and the potential development of antibiotic resistance (Owens *et al.*, 1997; Barton, 2000; Sol *et al.*, 2000; Erskine *et al.*, 2002; Makovec and Ruegg, 2003; Pitkala *et al.*, 2004; Pol and Ruegg, 2007). Another concern, discarded milk following antibiotic treatment may account for as much as 73% of lost marketable milk, and in herds that do not have a judicious treatment program, losses from discarded milk alone can exceed \$100 per cow in the herd per year (Bartlett, 1991).

It has been reported that 10 to 40% of cultures from CM cases yield no bacterial growth (NG) and so do not require antimicrobial therapy. Another 40% of positive cultures

(Gram-negatives, yeast) are not susceptible to most approved intramammary (IMM) products (Roberson *et al.*, 2003). Also, a high proportion of Gram-negative (GN) infections are quickly cleared by the cow's own immune system (although occasional persistence of GN infections occurs) (Pyörälä *et al.*, 1994; Erskine *et al.*, 1992). Conversely, intramammary (IMM) antibiotic therapy is routinely recommended for infections caused by Gram-positive (GP) organisms such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and environmental streptococci species. Based on these numbers, Roberson (2003) estimated that antibiotics labeled for IMM use would not be justified for 50 to 80% of CM cases.

Consequently, CM treatment decisions should be based on culture results. However, laboratory culture has not been routinely utilized by many dairies because of the time delay between submission of milk samples and reporting of results. Adoption of rapid on-farm milk culture (OFC) systems could allow producers to make strategic treatment decisions for CM cases, based on knowing the pathogen involved. The Minnesota Easy Culture System (University of Minnesota, Saint Paul, MN), a commercial OFC system, offers two different types of selective culture media systems. The Bi-plate system is a plate with two different types of agar: MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. Alternately, the Tri-plate system is a plate with three different types of agar. In addition to including MacConkey agar (GN growth) and Factor agar (GP growth), it also includes a section of MTKT agar which is selective for streptococci.

A previous paper has described that the selective treatment of clinical mastitis based in on-farm culture results reduces antibiotic use by half and tends to reduce milk withholding time without affecting short-term clinical and bacteriological cure outcomes (Lago *et al.*, 2009). However, long-term outcomes may also represent an important component of a clinical mastitis program economic impact. For example, in previous clinical mastitis in field trials comparing antibacterial and no antibacterial CM treatments, the risk for recurrence of CM did differ for mild CM cases where streptococci were isolated (Van Eenennam *et al.*, 1995). In addition, quarter milk SCC has been showed to be higher before and after CM (Sheldrake *et al.*, 1983; Schepers *et al.*, 1997; De Haas *et al.*, 2002). Likewise, CM results in milk production losses for the rest of the lactation (Houben *et al.*, 1993; Rajala-Schultz *et al.*, 1999; Wilson *et al.*, 2004; Gröhn *et al.*, 2008). In USA conventional production systems milk production losses associated with CM has been estimated to be over 500 kg over the entire lactation (Wilson *et al.*, 2004). However, because mature cows that suffer clinical mastitis are higher producers before the clinical event than their herdmates, the milk production losses over the entire lactation exceeds 1,000 kg over the entire lactation. Similarly, several studies have reported that clinical mastitis significantly increases the risk of culling (Dohoo and Martin, 1984; Erb *et al.*, 1985; Milian-Suazo *et al.*, 1989; Gröhn *et al.*, 1997; Rajala-Schultz *et al.*, 1999; Gröhn *et al.*, 2008). The culling hazard rate for CM cows was estimated to be more than twice that of non-CM cows (Gröhn *et al.*, 2008).

The objective of this study was to investigate the efficacy of using an OFC system to guide strategic treatment decisions in cows with mild and moderate CM, on long-term

outcomes in the same lactation including: a) risk and days to a recurrence of a CM event in the same quarter, b) somatic cell count (SCC), c) milk production, and d) cow survival post-enrollment (culling and death events).

MATERIALS AND METHODS

Study Design

A randomized controlled field trial was conducted between June 2005 and April 2007 in 8 dairy herds. In each herd cows were enrolled into the study for a period not longer than 6 months. These herds, 2 in Minnesota, 5 in Wisconsin and 1 in Ontario were a convenience sample of commercial dairy farms from the North American Great Lakes Region. Selected producers were required to maintain compliance with the study protocols and record keeping, trained personnel, individual animal identification, refrigeration and freezer capacity, participate in a Dairy Herd Improvement Association (DHIA) testing program, and demonstrate sufficient time and interest in the study. Herd size ranged from 150 to 1,800 cows, averaging 850 cows. Herds' housing systems, milk production and SCC are described elsewhere (Lago *et al.*, 2009).

Case Definition

Clinical mastitis was diagnosed if milk from one or more glands was abnormal in color, viscosity, or consistency, with or without accompanying heat, pain, redness, or swelling

of the quarter, or generalized illness. All lactating cows in the herd were eligible for enrollment at the time of occurrence of CM, except cows exhibiting severe or grade 3 CM (depression, anorexia, dehydration, fever), or any cow with fewer than three functional teats.

Enrollment Process

Cows with CM were detected in the milking parlor by the milkers upon appreciation of clinical signs of mastitis (e.g. visible abnormal milk and/or quarter). If the cow met the designated inclusion criteria for enrollment, herd personnel aseptically collected a single milk sample from the affected quarter. For a first CM episode (cow not previously enrolled into the study), eligible cows for enrollment were randomly assigned following a simple randomization schedule to either the positive-control group (PC) or culture-based treatment group (CB) by opening a pre-identified envelope following a sequential order. If more than one quarter was affected, all affected quarters were assigned to the same treatment group. For a second (or greater) CM episode in the same cow (i.e. cow had been previously enrolled), in the same or in a different quarter, the quarter was assigned to the same treatment group as was previously assigned.

Treatment Groups

Positive Control Group

Immediately after enrollment the quarter milk sample that had been collected was frozen on-farm at -20 °C and the affected quarter(s) was infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). The treatment was repeated once, 12 hours after the first treatment in according to label directions. A milk-withdrawal period of 96 hours and a slaughter withdrawal period of four days were followed after the last treatment.

Culture-Based Treatment Group

The aseptically collected milk sample(s) from the affected quarter(s) was first cultured on-farm using the Minnesota Easy Culture System (University of Minnesota, St. Paul, MN). This OFC system consists of a bi-plate which is a petri dish with two different types of agar, MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. A sterile cotton swab was dipped into the milk sample and then plated onto the Factor media half of the bi-plate, redipped into the milk, and then applied to the MacConkey media half of the bi-plate. The plate was placed in an on-farm incubator and incubated at approximately 37°C for 24 hours. The quarter milk sample that had been collected was then frozen on-farm at -20 °C. The next day the plate was read and interpreted according to guidelines provided for the Minnesota Easy Culture System. If bacteria did not grow, the plate was returned to the incubator and re-read approximately 24 hours later. Final results for each sample plate were recorded as a) GP, when bacteria grew only in the Factor agar media of the bi-plate, b) GN, when bacteria grew only in the MacConkey agar media of the bi-plate, c)

NG, when bacteria did not grow in either media, and d) mix infection when bacteria grew in both media. The decision about initiation of IMM antibiotic therapy the day after enrollment of the CM case was based on the on-farm culture results. Quarters from which GP bacteria were isolated or had a mix infection received the same IMM antibiotic treatment following the same procedures than cases assigned to PC. If the on-farm culture result was GN or NG, then the quarter did not receive IMM therapy.

Long term outcomes for the next 365 days or the end of the current lactation, which ever came first, recorded by farm personnel and captured through DHIA records included whether or not the quarter experienced a relapse of clinical mastitis, milk production, somatic cell count, and sale or death.

Laboratory Bacteriological Culture

Aerobic culture methodologies for frozen milk samples (enrollment day 0, day 14, day 21) collected on farms were standardized among labs at all three participating sites and performed in accordance with the National Mastitis Council guidelines (NMC, 1999).

Detailed laboratory procedures were described somewhere else (Lago *et al.*, 2009).

Data Analysis – Definition of Outcome Variables

Risk and Days to Recurrence of Clinical Mastitis in the Same Quarter

A recurrence was defined as detection of a new CM case in the same quarter at least 14 days after the previous enrollment case of CM. Cow ID, affected quarter(s), and date of recurrent CM cases was retrieved from dairy farm management or study records. Cows were followed until the end of the current lactation or 12 months after enrollment (whichever came first).

Somatic Cell Count and Milk Production

Monthly DHIA SCC and milk production records from individual cows were retrieved for the entire lactation from the on an-farm record keeping system (DairyComp305; Valley Agricultural Software, Tulare, CA). The test records used in this analysis were those up to 12 months after enrollment of the CM case. Milk SCC were log transformed to normalize the data to linear SCC (LSCC) using the linear SCC formula: $LSCC = [\text{LOGe}(\text{SCC})/0.6931] - 3.6439$ (Ali and Shook, 1980).

Risk and Days to Culling

For all cows in the study, the removal date (culling/death) was retrieved from the on an-farm record keeping system (DairyComp305; Valley Agricultural Software, Tulare, CA). Cows were followed until the end of the current lactation or up to 12 months after enrollment (whichever came first).

Statistical Analysis - Models and Modeling Strategy

Database summaries and plots were used for exploratory data analysis. Basic diagnostics techniques were used to evaluate normality, independence, homoscedasticity, collinearity and linearity of variables.

General Linear Mixed Models (GLMM) for Continuous Outcome Variables

Continuous outcome variables such as milk yield and LSCC were modeled as a function of explanatory variables using linear multivariable regression. A multilevel GLMM was constructed with milk yield / LSCC as a continuous, normally distributed response variable. The model was specified with random variation allowed in three hierarchical levels; repeated measure of milk yield / LSCC within cow, variation among cows within the herd, and variation among herds. This was accomplished with the MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) by specifying a correlation structure among the repeated measurements of the same cow, and including a random statement to account for clustering of cows within herds. In order to select the most appropriate covariance structure we started with a full model with all confounding covariates already taken into account. Summary statistics and exploratory data analysis plots to explore the covariance structure were created, and one model for each covariance structure was fitted. The correlation structures that were evaluated include simple (no correlation), compound symmetry, banded diagonal, autoregressive, and unstructured (estimating a correlation for each separate correlation). The different correlation structures were evaluated using

goodness of fit measures. The goodness of fit measures will include $-2 * \log$ likelihood ($-2LL$), Akaike's information criterion (AIC), and Bayesian information criterion (BIC).

Covariates such as cow parity, days in milk (DIM) at CM event, previous occurrence of a CM case in the same quarter in the present lactation, number of quarters affected, and etiology of infection were included in the model if it was a potential confounding variable. Other covariates such as LSCC in the test prior to the CM event were evaluated only when the estimated outcome was LSCC, and milk production in the test prior to the CM event when the estimated outcome was milk production. To determine if a covariate confounded the treatment effect on the outcome (milk yield or LSCC), the crude estimate of treatment group (PC vs. CB) was compared with the adjusted estimate for that third confounding variable. It was concluded that the variable confounded the association between treatment group and outcome variable if the ratio between the difference of the crude estimate and the adjusted estimate versus the crude estimate was greater than 10%. Each variable was examined for potential confounding one at a time by regression. Once the confounder variables were identified, the next step was to place all confounders into a full model with two-way interaction terms between treatment and the confounder. In order to simplify the model each non-significant interaction term was removed one at a time using a backward stepwise approach, starting by the least significant interaction term, and running the model again until there are no non-significant interaction terms in the model. Next, with non-significant interaction terms removed from the model, it was determined whether there were main effect variables in the model that were not in an interaction term that might be a confounder. The least significant term were removed and

it was evaluated if this affected the treatment effect estimate, with the goal to assess whether the variable confounded the treatment-outcome relationship. If the variable was an important confounder, it was returned to the model and other variables were assessed one at a time to see if they were confounders. The treatment variable was forced in the model regardless of the P -value. Once all non-significant interaction terms were removed as well as main effect variables that did not confound the exposure-outcome relationship, this was the final model. Final significance was declared at $P < 0.05$.

Time to Event Models

Binary responses with a 'time to event' component such as quarter risk and days to a CM recurrence or risk and days to removal from herd (culling/death) were modeled using survival analysis. Cox's proportional hazards regression method was used to test the logistic analysis explanatory variables (see previously described covariates) simultaneously for their association with time until event (PROC PHREG). The standard model was extended by including a frailty term reflecting a latent effect associated with each herd and with each cow when the event of interest was at the quarter level.

Cows (and quarters) were censored when the event of interest happened or at the end of a 12 months follow-up period (whichever occurred first). The assumption of independent censoring between both treatment groups was assessed by comparing the proportion of censored cows or quarters between both treatment groups. In addition, a sensitivity analysis looking at situations of complete positive correlation (every cow or quarter

censored experienced the event of interest) or negative correlation (censored cows or quarters did not experienced the event of interest) between censoring and the event of interest was done. If the violation of this assumption did not dramatically alter the treatment effect estimate (<10%), it was concluded that censoring did not introduce bias.

RESULTS

Descriptive Data

Four hundred and twenty two cows affected with CM in 449 quarters were enrolled in the study. Two hundred and fourteen cows with 229 affected quarters were assigned to PC, and 208 cows with 220 affected quarters were assigned to CB. Cow and quarter level descriptors and etiology of infection at enrollment for both study groups was described elsewhere (Lago *et al.*, 2009).

Clinical Mastitis Treatment Program Effects

Risk and Days to Recurrence of Clinical Mastitis

There were no significant differences in the risk and days to recurrence of a CM event between treatment programs [HR_{PC} (95% CI) = 1.2 (0.9, 1.6); $P = 0.2011$] (Table 3.1 and Figure 3.1). This risk was numerically higher in cases assigned to PC in 3 herds, the same in 1 herd, and lower in 3 herds of the 7 herds where risk and days to recurrence of a CM

event data was available. Clinical mastitis recurrence data was not available for one herd because of misplacement of follow-up CM cases records. Covariates that remained in the model because of confounding the treatment program effect on the risk and days to recurrence of CM included number of quarters affected and the occurrence of a previous case of CM in the same quarter in the present lactation.

The risk and average days after enrollment to recurrence of a CM event in the same quarter was 35% and 78 days or 43% and 82 days for cases assigned to PC and to CB, respectively. The overall risk and average days to recurrence of a CM event for NG, GN and GP cases were 41% and 81 days, 45% and 79 days, and 32% and 76 days, respectively.

Somatic Cell Count

There was no difference in LSCC after enrollment between treatment programs [Diff_{PC} (95% CI) = 0.1 (-0.2, 0.5); $P = 0.4142$] (Table 3.1 and Figure 3.1). The LSCC was numerically higher in cases assigned to PC in 3 herds, the same in 2 herds, and lower in 3 herds of the 8 herds enrolled in the study. No other covariates in addition to the explanatory variable of interest, treatment program, remained in the model because of confounding the treatment program effect on LSCC after enrollment. The autoregressive structure (AR), in which correlations between adjacent repeated somatic cell count measurements are higher than between measurements further apart, resulted in the best model fit, based on various goodness-of-fit measures.

The estimated LSCC for the rest of the lactation after enrollment was 4.2 and 4.4 for cows assigned to PC and to CB, respectively. The overall LSCC after enrollment for both CM treatment programs for NG, GN and GP cases were 3.9, 4.4 and 4.8, respectively.

Milk Production

There was no significant difference in milk production after enrollment between treatment programs [Diff_{PC} (95% CI) = 0.7 (-0.9, 2.3); P = 0.4431] (Table 3.1 and Figure 3.2). Milk production was numerically higher in cases assigned to PC in 4 herds and lower in 4 herds of the 8 herds enrolled in the study. No other covariates in addition to the explanatory variable of interest, treatment program, remained in the model because of confounding the treatment program effect on milk production after enrollment. The autoregressive structure (AR) also resulted in the best model fit, based on various goodness-of-fit measures.

The estimated daily milk production after enrollment was 30.0 and 30.7 kg for cows assigned to PC and to CB, respectively. The overall daily milk production after enrollment for both CM treatment programs for NG, GN and GP cases were 31.8, 30.6 and 28.8 kg, respectively.

Risk and Days to Removal from the Herd

There was no significant difference in the risk and days to removal from the herd due to culling or death, between treatment programs [HR_{PC} (95% CI) = 1.1 (0.7, 1.6); $P = 0.5604$] (Table 3.1 and Figure 3.3). This risk was numerically higher in cases assigned for the PC in 4 herds and lower in 4 herds of the 8 herds enrolled in the study. Covariates that remained in the model because of confounding the treatment program effect on the risk and days to removal included parity of the cow, DIM at enrollment and etiology of infection.

The risk and average days after enrollment to a culling or death event was 28% and 160 days and 32% and 137 days for cases assigned to PC and to CB, respectively. The overall risk and average days to a culling/death event for NG, GN and GP cases were 24% and 155 days, 28% and 127 days, and 36% and 175 days, respectively.

DISCUSSION

Risk for recurrence of a CM event in the same quarter was not different between both CM treatment programs. Similarly, in a clinical trial when comparing the CM recurrence risk between antibacterial and no antibacterial CM treatments, it did not differ for mild CM cases where no bacteria or coliforms were isolated (Van Eenennam *et al.*, 1995). However, in the present study the recurrence risk of a CM event for both CM treatment programs was higher than previously reported. It may be due to different infection pathophysiology characteristics or merely due to differences in the definition, detection and record keeping of CM cases among studies. Differences in etiology appears not to

explain the high recurrence risk since the risk and days to recurrence of a CM event was not different among the different CM etiology classification groups.

Milk production and LSCC after enrollment was not different between CM treatment programs. These results agreed with two previous clinical trials for which milk production did not differ between antibacterial and no antibacterial CM treatment groups for mild CM cases where no bacteria or coliforms were isolated (Van Eenennam *et al.*, 1995; Roberson *et al.*, 2004). However, another study reported that after resolution of CM, cows given antibiotics along with supportive treatment returned to normal performance, whereas cows given only supportive treatment alone incurred continued loss (Shim *et al.*, 2004). However, the latter study did not evaluate the effect of treatment on milk production depending on the etiology of the CM case. In addition, the authors hypothesized that the continued milk yield loss in CM cases that did not receive antibiotic therapy could be the result of more persistent subclinical infections or more marked alteration of mammary gland function. That was not the case in this study, since there were not differences between both treatment programs in the prevalence of IMI 14 and 21-d after the CM event (Lago *et al.*, 2009).

The risk and days to culling between both CM treatment programs was not different. These results agree with an earlier clinical trial for which the time to removal from the herd after CM did not differ between antibacterial and no antibacterial treatment groups nor by etiology of infection (Van Eenennam *et al.*, 1995).

It has been reported in an accompanying manuscript that the use of an OFC system to guide the strategic treatment of CM reduced IMM antibiotic use by half and tended to reduce in one day the milk withholding time, without significant differences in short-term clinical and bacteriological outcomes after CM (Lago *et al.*, 2009). Additionally, long-term outcomes of the intervention were evaluated in this manuscript since they may represent an important component of the overall economic impact of the intervention. The recurrence risk of clinical mastitis appears to be a sensitive indicator of differences in treatment efficacy between treatments. For example in previous trials, the recurrence risk of clinical mastitis did differ for antibacterial and no antibacterial CM treatment groups, for mild CM cases where streptococci were isolated (Van Eenennam *et al.*, 1995). Similarly, several studies reported that after CM, quarter milk SCC is higher (Sheldrake *et al.*, 1983; Schepers *et al.*, 1997; De Haas *et al.*, 2001), milk production is lower (Houben *et al.*, 1993; Rajala-Schultz *et al.*, 1999; Wilson *et al.*, 2004; Gröhn *et al.*, 2008), and the risk of culling is higher (Dohoo and Martin, 1984; Erb *et al.*, 1985; Milian-Suazo *et al.*, 1989; Gröhn *et al.*, 1997; Rajala-Schultz *et al.*, 1999; Gröhn *et al.*, 2008). Consequently, these outcomes must be evaluated in order to compare the overall biologic and economic impact of treatment interventions for CM.

The strengths of the present study are related to the validation of a program to treat clinical mastitis. The success of the CB program not only depends on the inefficacy of antibiotic IMM treatment in NG or GN cases, but also depends on the accuracy of the OFC system, and the effects of a one day delay to initiate IMM antibiotic therapy in those quarters selected for treatment. The label of IMM antibiotic administered in this study,

Cefa-Lak[®] (Fort Dodge Animal Health, Fort Dodge, IA) does not include efficacy claims against Gram-negative bacteria. Two IMM antibiotic preparations, Hetacin-K[®] (Fort Dodge Animal Health, Fort Dodge, IA) and Spectramast[®] (Pfizer Animal Health, New York, NY), are currently approved in the USA with a label that claims efficacy against clinical mastitis in lactating dairy cattle associated with *Escherichia coli*. The validity of the present study results when used with antibiotics other than Cephapirin Sodium is not known.

CONCLUSIONS

It has been reported elsewhere that the selective treatment of clinical mastitis based in OFC results halves antibiotic use and tends to reduce milk withholding time without affecting short-term clinical and bacteriological outcomes (Lago *et al.*, 2009). The present article reports no differences in long-term outcomes such as recurrence of clinical mastitis in the same quarter, somatic cell count, milk production, and cow survival for the rest of the lactation after CM. Results of both analyses will be used to evaluate the overall cost-benefit of using an on-farm culture system to guide strategic treatment decisions in cows with mild and moderate CM.

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TABLES

Table 3. 1. Clinical mastitis recurrence, somatic cell count, daily milk yield and culling for two clinical mastitis treatment programs (long-term outcomes).

Parameter	Positive-Control		Culture-Based		Treatment Effect	
	Cows ^a	Herds ^b	Cows	Herds	Estimate (95% CI)	P-value
Recurrence of Clinical Mastitis [% (n)] – Quarter Level						
No growth	34 (68)		48 (73)			
Gram-negatives	39 (56)		52 (42)			
Gram-positives	29 (68)		34 (79)			
All cases	35 (220)	23-40	43 (210)	22-63	HR _{PC} = 1.2 (0.9, 1.6)	0.2011
Linear Somatic Cell Count [% (n)] – Cow Level						
No growth	3.9 ± 0.2 (57)		4.0 ± 0.2 (60)			
Gram-negatives	4.3 ± 0.3 (48)		4.6 ± 0.3 (44)			
Gram-positives	4.7 ± 0.3 (52)		4.9 ± 0.2 (63)			
All cases	4.2 ± 0.1 (178)	3.2-5.8	4.4 ± 0.1 (178)	3.4-5.4	Diff _{PC} = 0.1 (-0.2, 0.5)	0.4431
Daily Milk Yield [Mean ± SD (n)] – Cow Level						
No growth	32.2 ± 1.0 (57)		31.4 ± 1.0 (60)			
Gram-negatives	30.0 ± 1.1 (48)		31.5 ± 1.3 (44)			
Gram-positives	29.1 ± 1.1 (52)		28.6 ± 1.1 (63)			
All cases	30.0 ± 0.6 (178)	26-33	30.7 ± 0.6 (178)	27-39	Diff _{PC} = 0.7 (-0.9, 2.3)	0.4142
Culling / Death [Mean ± SD (n)] – Cow Level						
No growth	22 (58)		26 (65)			
Gram-negatives	30 (50)		25 (36)			
Gram-positives	37 (49)		35 (62)			
All cases	28 (195)	19-60	32 (195)	11-43	HR _{PC} = 1.1 (0.7, 1.6)	0.5604

^a Cow and quarter level descriptors

^b Herds range for the different descriptors [min herd (%) – max herd(%)]

Figure 3. 1. Kaplan-Meier survival graph representing the probability of a recurrence of a clinical mastitis case in the same quarter at a given days after the clinical mastitis event for two clinical mastitis treatment programs. Clinical mastitis cases assigned to the positive-control treatment program are represented by a solid line and cases assigned to the culture-based treatment program are represented by a dashed line.

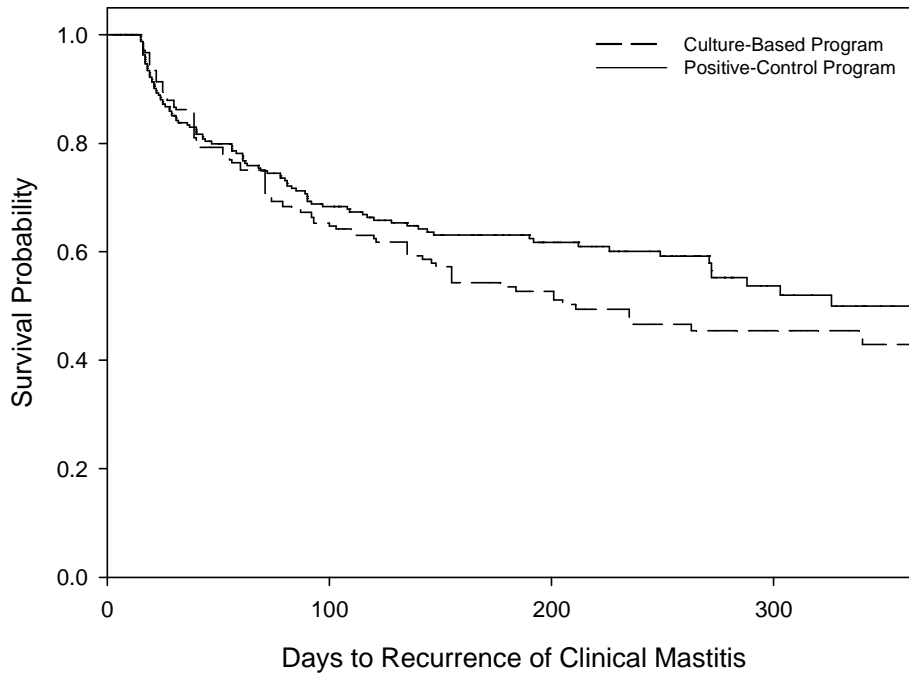


Figure 3. 2. Kaplan-Meier survival graph representing the probability of culling or death at a given days after the clinical mastitis event for two clinical mastitis treatment programs. Cows with clinical mastitis assigned to the positive-control treatment program are represented by a solid line and cows assigned to the culture-based treatment program are represented by a dashed line.

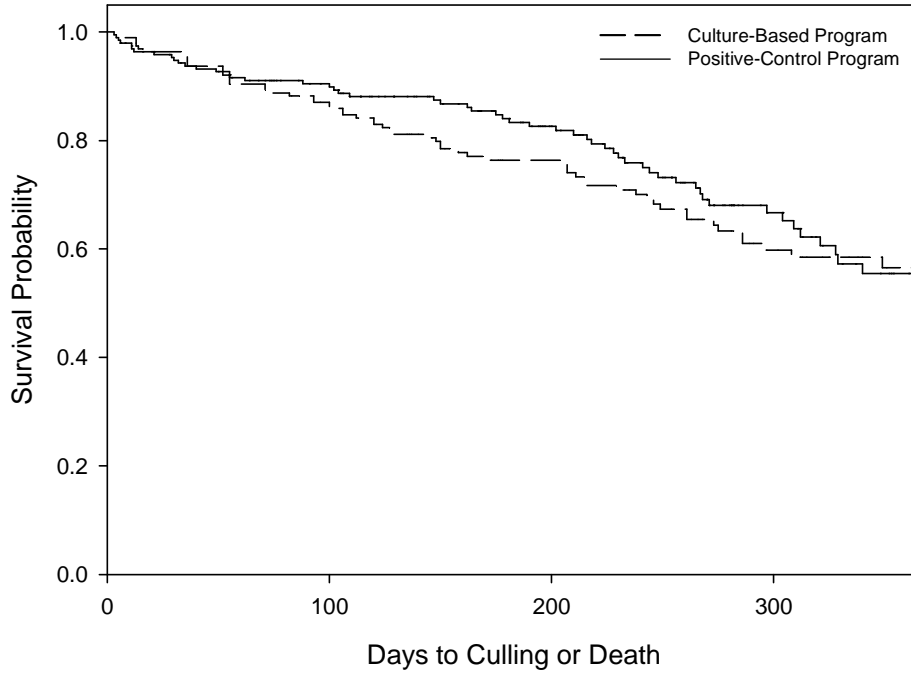


Figure 3. 3. Least square LSCC mean up to ten DHIA tests after the clinical mastitis event for two clinical mastitis treatment programs. Cows with clinical mastitis assigned to the positive-control treatment program are represented by a solid line and cows assigned to the culture-based treatment program are represented by a dashed line.

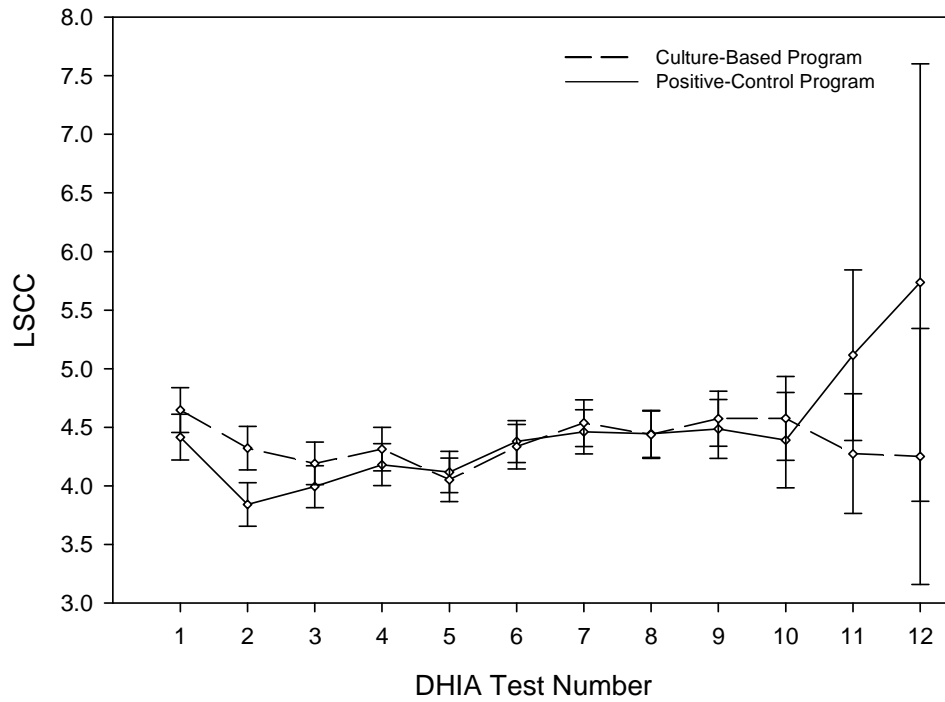
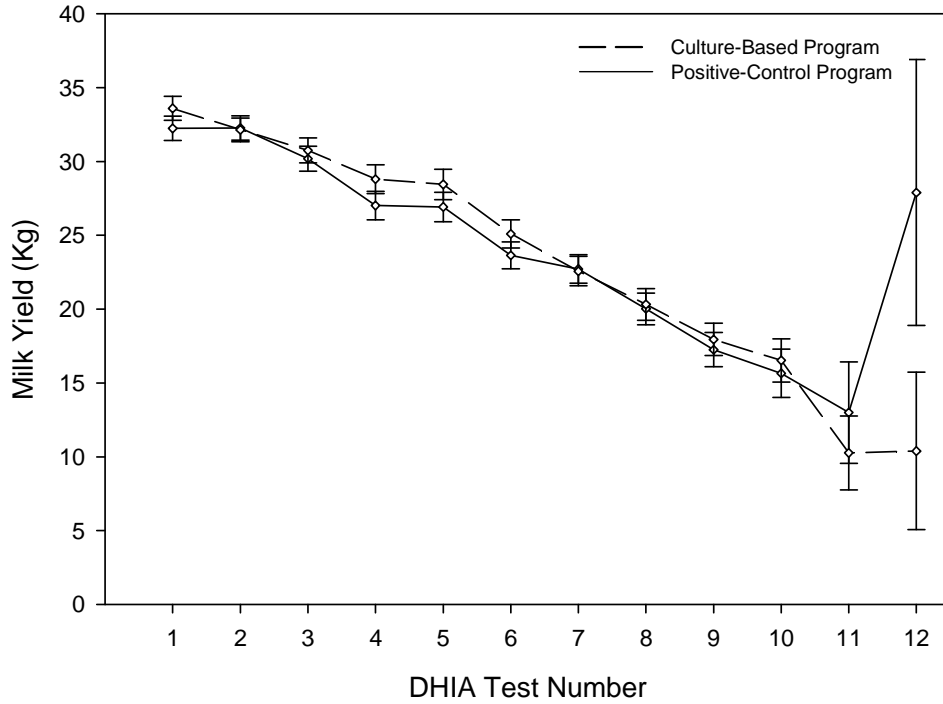


Figure 3. 4. Least square milk yield mean up to ten DHIA tests after the clinical mastitis event for two clinical mastitis treatment programs. Cows with clinical mastitis assigned to the positive-control treatment program are represented by a solid line and cows assigned to the culture-based treatment program are represented by a dashed line.



CHAPTER IV

EFFICACY OF TWO PROGRAMS DESIGNED TO DIAGNOSE AND TREAT SUBCLINICAL INTRAMAMMARY INFECTIONS AFTER PARTURITION ON ANTIBIOTIC USE, DAYS OUT OF THE TANK AND BACTERIOLOGICAL OUTCOMES

The objective of this multi-state multi-herd clinical trial was to investigate the efficacy of using the California Mastitis Test (CMT) alone, or the CMT and an on-farm culture test in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition. A total of 1,885 cows from 14 herds were screened for enrollment into the study at 1-4 days after parturition. Of those, 1,168 cows which had a negative CMT result on all four quarters were not assigned to any treatment group. A total of 717 cows with at least one CMT-positive quarter were randomly assigned to either a) a negative-control group (NC), b) a CMT-based treatment group (CMTB), or c) to a culture-based treatment group given a CMT-positive result (CB|CMT-pos). Quarters from cows assigned to NC did not receive IMM antibiotic treatment. CMT-positive quarters from cows assigned to CMTB received immediate on-label intramammary treatment with Cephapirin Sodium. Quarters from cows assigned to CB|CMT-positive were not treated until the results of on-farm culture were determined after 24 hr of incubation. Quarters from cows assigned to CB|CMT-positive showed Gram-positive growth were treated according to label instructions using intramammary Cephapirin Sodium. Quarters assigned to CB|CMT-

positive showed no growth, Gram-negative or a mixed infection did not receive intramammary therapy. The risk to receive intramammary antibiotic therapy for CMT-positive cows and quarters assigned to CMTB was 100 and 50%, respectively. The risk to receive intramammary antibiotic therapy for CMT-positive cows and quarters assigned to CB|CMT-pos was 40 and 15%, respectively. There was an increase in the days of milk withheld from the market for cows in both study groups where intramammary antibiotics were used, 6.3 days for CMTB [Diff_{NC} (95% CI) = 4.7 (4.4, 5.0); P < 0.0001] and 4.4 days for CB|CMT-pos [Diff_{NC} (95% CI) = 2.5 (2.0, 3.0); P < 0.0001], compared to 1.7 days for NC. The odds for a bacteriological cure within 21 days of enrollment were significantly higher for quarters of cows assigned to CMTB [OR_{NC} (95% CI) = 2.4 (1.5, 3.7); P = 0.0002] and tended to be higher for quarters of cows assigned to CB|CMT-positive [OR_{NC} (95% CI) = 1.5 (0.9, 2.4); P = 0.07]. The proportion of quarters with bacteriological cure was 59, 50 and 42% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively. Using NC as the reference, there was no significant difference in the risk for new IMI for quarters from cows assigned to CMTB [OR_{NC} (95% CI) = 1.0 (0.8, 1.3); P = 0.99] and to CB|CMT-pos [OR_{NC} (95% CI) = 1.0 (0.8, 1.2); P = 0.97]. The proportion of quarters with a new IMI was 44, 46 and 45% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the ICR risk (where ICR risk represented the presence of infection risk, CM risk, or removal from herd risk within 21 days after enrollment) for quarters from cows assigned to CMTB [OR_{NC} (95% CI) = 0.8 (0.6, 1.1); P = 0.23] and to CB|CMT-pos [OR_{NC} (95% CI) = 0.8 (0.6, 1.1); P = 0.23]. The ICR risk was 53, 58 and 59% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

INTRODUCTION

Subclinical intramammary infection (IMI) at calving is a common occurrence. Reported prevalence of quarter IMI at parturition in North-American studies ranges from 29% to 63% (Fox *et al.*, 1994; Roberson *et al.*, 1994; Kirk *et al.*, 1996; Oliver *et al.*, 1997; Sargeant *et al.*, 2001; Godden *et al.*, 2003; Wallace *et al.*, 2004). A negative relationship between early lactation somatic cell count (SCC) and SCC, milk production and culling hazard during the first lactation has been established in heifers (De Vliegher *et al.*, 2004; De Vliegher *et al.*, 2005a; De Vliegher *et al.*, 2005b). Consequently, there is a need to prevent these infections from occurring, and to develop treatment strategies to diminish the disease impact. One potential approach to address this goal may be to develop programs to identify and treat (cure) subclinical IMI present at the beginning of the lactation.

One such strategy, the use of intramammary (IMM) antibiotic therapy to treat IMI in the prepartum period, is controversial. A series of trials evaluating the efficacy of prepartum IMM antibiotic therapy on subclinical mastitis in heifers during early lactation concluded that prepartum IMM antibiotic infusion at 7 or 14 d before expected parturition is an effective procedure for eliminating many infections in heifers during late gestation, and for reducing the prevalence of mastitis in heifers during early lactation and throughout lactation (Oliver *et al.*, 1992; Oliver *et al.*, 1997; Oliver *et al.*, 2003; Oliver *et al.*, 2004). In that study, prepartum antibiotic-treated heifers had a lower prevalence of mastitis

pathogen isolation throughout lactation, produced significantly more milk than control heifers and had significantly lower SCC than untreated control heifers. However, a more recent multi-state, multi-herd study reported that, while prepartum IMM antibiotic therapy did reduce the number of heifer IMI postpartum, milk production, SCC, and reproductive performance during the first 200 d of the first lactation were not affected by treatment (Borm *et al.*, 2006).

Another strategy to identify and treat subclinical IMI in periparturient cows could involve a cowside screening tool, the California mastitis test (CMT), a qualitative measurement of the SCC in milk. Recent studies using the CMT test in the first week after calving have suggested that there may be potential for its use as a screening tool to identify subclinical IMI in fresh cows (Sargeant *et al.*, 2001; Wallace *et al.*, 2002; Dingwell *et al.*, 2003). However, a high false positive rate was reported. Dingwell *et al.* (2003) reported that if the CMT yielded a negative result, the producer could be 95% certain the quarter was truly uninfected. However, if the test yielded a positive result, there was a 79% chance that it was a false-positive result.

Controlled studies evaluating the efficacy of antibiotic therapy for IMI based on CMT testing in early lactation are scarce, and the ones carried out have reported mixed results. Rosenberg evaluated cure rates and SCC when CMT-positive quarters in cows after parturition were treated with IMM Cephapirin Sodium (Rosenberg *et al.*, 2002). It was determined that by four weeks post-calving, quarters treated with Cephapirin Sodium had significantly increased cure rates and SCC were significantly reduced compared with

untreated control quarters. Conversely, Wallace *et al.* (2004), also randomly assigning cows with CMT-positive quarters to receive either IMM Cephapirin Sodium or no treatment, found that there was no difference in cure rates for IMM antibiotic-treated quarters for major pathogens compared to the untreated controls.

These early studies suggest that limitations of using the CMT test alone as a screening tool to identify IMI include i) poor test specificity in fresh cows, and ii) the CMT test cannot give information on the likely etiology of infection. Since many mastitis researchers consider that IMM antibiotic therapy may only be indicated in the case of IMI caused by Gram-positive pathogens, then using the CMT test alone could result in unnecessary and inappropriate treatment of many fresh cows (Wallace *et al.*, 2004). Due to these limitations, it has been suggested that a rapid and accurate on-farm confirmatory test is needed to verify the presence and etiology of IMI present in CMT-positive quarters of fresh cows. On-farm culture (OFC), a rapid and inexpensive on-farm diagnostic tool, could conceivably be used as a confirmatory test, in conjunction with a screening tool (such as CMT), to diagnose and guide strategic treatment of subclinical IMI in cows after parturition.

The objective of this study was to investigate the efficacy of using the CMT alone, or CMT and an OFC system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition. Outcomes evaluated included: a) quarter and cow risk to receive IMM antibiotic therapy because of study assignment, b) days of milk withheld from market (days out of the tank), c) bacteriological cure within 21 days of

enrollment, d) new intramammary infection (IMI) risk within 21 days of enrollment, and e) presence of infection, risk of clinical mastitis, or risk of removal from herd (ICR) within 21 days of enrollment.

MATERIALS AND METHODS

Study Design

A randomized controlled field trial was conducted between June 2005 and August 2006 in 14 Holstein herds. In each herd cows were enrolled into the study for a period not longer than 6 months. Study herds, 5 in Minnesota, 1 in Wisconsin and 8 in Ontario, were a convenience sample of commercial dairy farms from the North American Great Lakes Region. Selected producers were required to maintain compliance with the study protocols and record keeping, have trained personnel, individual animal identification, treatment facilities, appropriate drug storage capabilities, refrigeration and freezer capacity, participate in a Dairy Herd Improvement Association (DHIA) testing program, and compliance. Herd size ranged from 40 to 4,000 cows (median 356 cows). Eight and six of the herds utilized free-stalls and tie-stall housing systems, respectively. Annual milk production among those herds ranged from 8,600 kg to 12,600 kg, averaging 10,600 kg. Bulk tank milk SCC ranged from 125,000 cells/ml to 432,000 cells/ml, averaging 224,000 cells/ml.

Enrollment Process

All cows in the first three days after calving were eligible for enrollment unless they exhibited signs of clinical mastitis at time of calving, or had fewer than three functional teats. If the cow met the designated inclusion criteria for enrollment, the herdsman aseptically collected a single milk sample from all four quarters and performed the CMT on individual quarters. If the cow failed to meet the designated enrollment criteria because of clinical mastitis, then a single milk sample was still collected from the affected quarter(s).

California Mastitis Test

Herd personnel performed the CMT on individual quarters by stripping milk from each quarter into of each of the four wells in the CMT paddle. The paddle was slightly tipped to pour off any excess milk and to create an equal volume of milk, approximately 2 ml, in each of the 4 wells. Next, an equal volume of CMT solution was squirt into each of the 4 wells, creating an approximate 1:1 mixture of milk and CMT solution. Finally, the paddle was gently rotated and swirled for 5 seconds to mix milk and CMT solution. The interpretation of the CMT results were as follows: a) negative when there was no gel formation; b) 1+ when there was light or mild thickening (includes trace); c) 2+ when moderately thick gel formed; and d) 3+ when a very thick gel was formed (center became elevated like a fried egg). CMT results for each one of the all four quarters were recorded. This use and interpretation of the CMT was in accordance with the NMC (1999) recommendations.

Allocation to Treatment Group

For cows with a negative CMT result on all four quarters no treatment group was assigned and no further action was taken with the cow. Previously collected quarter milk samples were frozen. Cows with at least one CMT-positive quarter were randomly assigned following a simple randomization schedule to either a negative-control group (NC), a CMT-based treatment group (CMTB), or to a culture-based treatment group given a CMT-positive result (CB|CMT-positive) by opening a pre-identified envelope following a sequential order.

Treatment Groups

Negative Control Group

Quarter milk samples that had been collected from all quarters from the cow were frozen on-farm at -20 °C. Quarters from cows assigned to the NC group did not receive IMM antibiotic treatment.

CMT-Based Treatment Group

Immediately after enrollment the quarter milk samples that had been collected from all quarters from the cow were frozen on-farm at -20 °C. CMT-positive quarters were then

infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). The treatment was repeated once, 12 hours after the first treatment, according to label directions. A milk-withdrawal period of at least 96 hours and a slaughter withdrawal period of four days were followed after the last treatment.

Culture-Based given a CMT-Positive Result Treatment Group

The aseptically collected milk sample(s) from CMT-positive quarter(s) were first cultured on-farm using the Minnesota Easy Culture System (University of Minnesota, St. Paul, MN). This OFC system consists of a bi-plate which is a petri dish with two different types of agar, MacConkey agar on one half selectively grows Gram-negative organisms, while Factor agar on the other half of the plate selectively grows Gram-positive organisms. A sterile cotton swab was dipped into the milk sample and then plated onto the Factor media half of the bi-plate, redipped into the milk, and then applied to the MacConkey media half of the bi-plate. The plate was placed in an on-farm incubator and incubated at approximately 37°C for 24 hours. The quarter milk sample that had been collected was then frozen on-farm at -20 °C. The next day the plate was read and interpreted according to guidelines provided for the Minnesota Easy Culture System. If bacteria did not grow, the plate was returned to the incubator and re-read approximately 24 hours later. Final results for each sample plate were recorded as a) Gram-positive (GP), when bacteria grew only in the Factor agar media of the bi-plate, b) Gram-negative (GN), when bacteria grew only in the MacConkey agar media of the bi-plate, c) No growth (NG), when bacteria did not grow in either media, and d) mix infection when

bacteria grew in both media. The decision about initiation of IMM antibiotic therapy the day after enrollment was based on the on-farm culture results. Quarters from which GP bacteria were isolated received the same IMM antibiotic treatment following the same procedures than quarters from cows assigned to CMTB. If the on-farm culture result was GN, NG or a mix infection, then the quarter did not receive IMM therapy.

After enrollment farm personnel recorded the date and time when IMM antibiotic treatments were administered and the number of days out of tank after parturition. Study technicians visited the study herds once per week and aseptically collect single quarter milk samples from all quarters of enrolled cows at approximately 14 days post-enrollment (10-16 days post-enrollment) and 21 days post-enrollment (17-23 days post-enrollment). All milk samples were transported on ice to the regional mastitis culture laboratory (St. Paul, MN; Madison, WI or Guelph, ON) and frozen at -20 °C until bacteriological culture was completed.

Laboratory Bacteriological Culture

Aerobic culture methodologies for frozen milk samples (enrollment day 0, day 14, day 21) collected on farms were standardized among labs at all three participating sites and performed in accordance with the National Mastitis Council guidelines (NMC, 1999). Briefly, individual quarter milk samples were thawed at room temperature. While still cold, 0.01 ml of milk was plated onto MacConkey agar and Factor agar using sterile calibrated loops. Factor Agar, similar to KLMB agar (Beatty *et al.*, 1985), selects for GP

organisms while inhibiting the growth of GN bacteria with antibiotics. Inoculated plates were incubated at 37°C. After incubation for 18 to 24 h, all plates were observed for microbial growth. Those plates having growth were recorded and species identification started. All plates were placed in the incubator for an additional 36 to 48 h and reevaluated for microbial growth. Colonies on MacConkey agar plates were presumptively identified based on colony morphology. Colony color was used as a means of determining if the organism on the plate was a lactose-fermenting organism. Isolates were also Gram stained to assist in organism identification. Organism identity was confirmed using the API 20E test (bioMerieux-Vitek, Inc. Hazelwood, MO). Colonies suspected as being staphylococci based on morphology were confirmed as staphylococci based on catalase reaction and microscopic morphology. Organisms suspected of being *Staphylococcus aureus* were confirmed using the tube coagulase reaction. Those organisms that were catalase-positive and coagulase-negative were classified as *Staphylococcus* spp. Catalase-negative streptococci were streaked onto TKT medium, which is selective for *Streptococcus* spp. only, to determine the esculin reaction and presumptive identification prior to organism confirmation using the API Streptococcus identification system (bioMerieux-Vitek, Inc. Hazelwood, MO).

Data Analysis – Definition of Outcome Variables

Risk to Receive IMM Antibiotic Therapy

None of the CMT-pos quarters from cows assigned to NC were treated, all CMT-positive quarters from cows assigned to CMTB were treated with two infusion syringes of (200

mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). However, for CMT-positive quarters from cows assigned to CB|CMT-positive only quarters from which GP bacteria were isolated received antibiotic treatment initially. Whether a cow or a quarter was treated with IMM antibiotics was recorded as dichotomous variable (Yes/No).

Days Out of the Tank

The date and time when milk was first marketed after parturition was also recorded by herd personnel. Number of days out of the tank was a continuous variable calculated as difference between parturition date and date milk was marketed.

Bacteriological Cure Risk

A quarter was considered infected when one or two bacterial species were isolated from a quarter milk sample in the laboratory. The isolation of two bacterial species in the laboratory was considered a mixed infection. A quarter sample was considered contaminated if three or more bacterial pathogens were isolated in the laboratory. A bacteriological cure (Yes/No) within a quarter was defined as the presence of one or two microorganisms in the enrollment milk sample, and the absence of the same specified microorganism(s) in both d-14 and d-21 milk samples. A cow was considered cured (Yes/No) if all infected quarters cured.

New IMI Risk

A quarter was considered newly infected (Yes/No) whenever a new bacterial species that was not previously present in the enrollment sample (d-0) was isolated from quarter milk samples collected either at d-14 or d-21 after enrollment based on in-lab culture. A cow with a quarter new IMI was considered newly infected (Yes/No).

ICR Risk

The presence of infection, occurrence of clinical mastitis, or removal from herd because of culling or death in the first 21 days after parturition described as a dichotomous outcome ($Y = 0$ or 1) called ICR risk. Presence of infection ($I =$ infection) represented the absence (0) or presence (1) of infection in quarter milk samples collected either at d-14 or d-21 samples after enrollment. Quarters that could not be resampled at d-14 or d-21 after enrollment because the quarters experienced a clinical mastitis event ($C =$ Clinical) during the follow-up period were assigned an ICR risk value of 1 . Similarly, quarters that could not be resampled at d-14 or d-21 because cows were removed from the herd because of culling or death ($R =$ Removed) were assigned an ICR risk value of 1 . Analysis of the parameter called ICR risk ($1/0$) was done in an attempt to eliminate potential omission bias created by not including, in the bacteriological cure analysis, quarters where no bacteria were isolated from the enrollment sample, and quarters without a follow-up culture result because of a clinical mastitis event or because the cow was removed from the herd.

Statistical Analysis

Database summaries and plots were used for exploratory data analysis. Basic diagnostics techniques were used to evaluate normality, independence, homoscedasticity, collinearity and linearity of variables. Different models were used to evaluate the CMTB and CB|CMT-positive treatment programs effect on the outcomes of interest using records from cows assigned the NC group as the reference.

Generalized Linear Mixed Models for Dichotomous Outcome Variables

Binary response variables such as quarter and cow risk for a bacteriological cure, quarter and cow risk for a new IMI, and quarter and cow ICR risk were modeled as a function of treatment group and other covariates using logistic multivariable regression. The treatment effect on the risk for the listed outcome variables following enrollment was analyzed by generalized linear mixed models using the GLIMMIX PROC of SAS version 9.1 (SAS Institute, Cary, NC) with cow (when the outcome was at the quarters level) and herd included as random effects.

General Linear Mixed Models for Continuous Outcome Variables

Days out of tank, a continuous outcome variable, was modeled as a function of explanatory variables using linear multivariable regression. A multilevel model was

constructed with days out of tank as a continuous, normally distributed response variable. The model was specified with random variation allowed in two hierarchical levels; variation among cows within the herd, and variation among herds. This was accomplished with the MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) by including a random statement to account for clustering of cows within herds.

Covariates such as cow parity, quarter CMT result, number of quarter(s) from the same cow from which bacteria was isolated at the laboratory, and etiology of infection were included in the model if it was a potential confounding variable. To determine if a covariate confounded the treatment effect on the outcome, the crude estimate of treatment group (CMTB *vs.* NC) and (CB|CMT-positive *vs.* NC) was compared with the adjusted estimate for that third confounding variable. It was concluded that the variable confounded the association between treatment group and outcome variable if the ratio between the difference of the crude estimate and the adjusted estimate versus the crude estimate was greater than 10%. Each variable was examined for potential confounding one at a time by regression. Once the confounder variables were identified, the next step was to place all confounders into a full model with two-way interaction terms between treatment and the confounder. In order to simplify the model each non-significant interaction term was removed one at a time using a backward stepwise approach, starting by the least significant interaction term, and running the model again until there were no non-significant interaction terms in the model. Next, with non-significant interaction terms removed from the model, it was determined whether there were main effect variables in the model that were not in an interaction term that might be a confounder.

The least significant terms were removed and it was evaluated if this affected the treatment effect estimate, with the goal to assess whether the variable confounded the treatment-outcome relationship. If the variable was an important confounder, it was returned to the model and other variables were assessed one at a time to see if they were confounders. The treatment variable was forced in the model regardless of the *P*-value. Once all non-significant interaction terms were removed as well as main effect variables that did not confound the exposure-outcome relationship, this was the final model. Final significance was declared at $P < 0.05$.

RESULTS

Descriptive Data

A total of 1,885 cows were enrolled in the study. Of those, 1,168 had a negative CMT result on all four quarters, which were not assigned to any treatment group, and 717 cows with at least one CMT-positive quarter which were randomly assigned to the NC, CMTB or CB|CMT-positive study groups. Two hundred and forty one cows were assigned to NC, 232 cows to CMTB, and 244 cows were assigned to CB|CMT-positive. Cow and quarter level descriptors and etiology of infection at enrollment for the three study groups are shown in Table 4.1. The parity distribution of the cows enrolled was 46%, 25% and 29% for first, second and third or greater parity cows, respectively. The mean and median DIM at enrollment was 1.5 and 1 days, respectively. The quarter CMT score distribution for cows with at least one CMT-positive quarter enrolled in the study was 48% negative,

31% 1+, 14% 2+ and 7% 3+. As a result, the average number of CMT-positive quarters for cows with at least one CMT-positive quarter was 1.9.

Bacteria were isolated from 43% of quarters from CMT-positive cows at enrollment. Coagulase-negative staphylococci bacteria were the most commonly isolated pathogen (27% of quarters), followed by non-agalactiae streptococci (5% of quarters), *Bacillus* spp. (5% of quarters), *Staphylococcus aureus* (1% of quarters), and other infections (1% of quarters). Gram-negative bacteria were isolated from only 2% of the quarters, with *E. coli* isolated from 1% of all quarters. Other GN isolates including *Klebsiella* spp., *Enterobacter* spp., and *Serratia* spp. represented 1% of all quarters. *Streptococcus agalactiae* was not isolated from any of the quarters. The non-agalactiae streptococci including *Enterococcus* spp., *Streptococcus uberis*, *Aerococcus* spp. and *Streptococcus dysgalactiae* represented 2%, 1%, 1% and less than 1% of all quarters. Other bacteria such as *Arcanobacterium pyogenes*, *Corynebacterium bovis*, and *Citrobacter* spp. each represented less than 1% of quarters.

Treatment Programs Effects

Risk to Receive IMM Antibiotic Therapy because of Study Assignment

The risk to receive IMM antibiotic therapy for cows and quarters assigned to CMTB was 100 and 50%, respectively (Table 4.2). The quarter risk ranged from 31 to 75% for the 14 dairy herds enrolled in the study. At the herd level (including in the denominator of the

proportion the CMT-negative cows) the risk to receive IMM antibiotic therapy for cows and quarters assigned to CMTB was 37 and 19%, respectively (Table 4.1).

The risk to receive IMM antibiotic therapy for cows and quarters assigned to CB|CMT-pos was 40 and 15%, respectively (Table 4.2). The cow risk ranged from 0 to 100% and the quarter risk ranged from 0 to 27% for the 14 dairy herds enrolled in the study. At the herd level (including in the denominator of the proportion the CMT-negative cows) the risk to receive IMM antibiotic therapy for cows and quarters assigned to CB|CMT-pos was 15 and 6%, respectively (Table 4.2). The etiologic classification distribution based in OFC for CMT-positive quarters assigned to CB|CMT-pos was 60% NG, 4% GN and 3% mix infections (did not receive IMM antibiotic therapy), and the 33% of the CMT-positive quarters were GP (received IMM antibiotics).

Days out of the Tank

The mean and median days of milk withheld from the market for CMT-positive cows assigned to NC (cows did not receive IMM treatment in any quarter) was 1.7 and 2.0 days. This time was 6.3 and 6.0 days for CMT-positive cows assigned to CMTB (all cows received IMM treatment in CMT-positive quarters) [Diff_{NC} (95% CI) = 4.7 (4.4, 5.0); $P < 0.0001$] (Table 4.2). This mean time ranged from 4.0 to 8.3 days for the 14 dairy herds enrolled in the study. No other covariates in addition to the explanatory variable of interest, treatment program, remained in the model because of confounding the treatment program effect on days of milk withheld from the market.

The mean and median days of milk withheld from the market for CMT-positive cows assigned to CB|CMT-pos (cows received IMM treatment if GP bacteria was cultured on-farm from CMT-positive quarters) was extended to 4.4 and 3.0 days [Diff_{NC} (95% CI) = 2.5 (2.0, 3.0); P < 0.0001] (Table 4.2). This mean time ranged from 1.8 to 7.3 days for the 14 dairy herds enrolled in the study. This time was 7.8 days for cows that received IMM treatment and 2.0 days for not treated cows. Other than the variable describing treatment program, no other covariates remained in the model.

Quarter Milk Bacteriological Culture Follow-Up

Bacteriological Cure Risk

The proportion of quarters with bacteriological cure was 42% and 59% for quarters of cows assigned to NC and to CMTB, respectively. This quarter-level risk for a bacteriological cure was higher for quarters of cows assigned to CMTB than for quarters of cows assigned to NC [OR_{NC} (95% CI) = 2.4 (1.5, 3.7); P = 0.0002] (Table 4.3). The only covariate that remained in the quarter-level model because of confounding the treatment program effect on bacteriological cure risk was etiology of infection. At the cow level, the proportion of cows with bacteriological cure was 35% and 47% for cows assigned to NC and to CMTB, respectively [OR_{NC} (95% CI) = 1.6 (0.9, 2.7); P = 0.1186] (Table 4.3). The only covariate that remained in the cow-level model because of confounding the treatment program effect on bacteriological cure risk was number of

quarter(s) from the same cow from which bacteria was isolated at the laboratory.

Although it was not a stated objective of this study, it was interesting to observe that among quarters within cows assigned to CMTB, the bacteriological cure risk for infected quarters that received IMM antibiotic treatment (CMT-positive quarters) was 61% and for infected quarters that did not receive IMM antibiotic treatment (CMT-negative quarters) was 57%.

The proportion of quarters with bacteriological cure was 42% and 50% for quarters from cows assigned to NC and to CB|CMT-pos, respectively. This represented a trend for a higher risk for a bacteriological cure for quarters of cows assigned to CB|CMT-pos than for quarters of cows assigned to NC [OR_{NC} (95% CI) = 1.5 (0.9, 2.4); P = 0.0782] (Table 4.3). The only covariate that remained in the quarter-level model because of confounding of the treatment program effect on bacteriological cure risk was etiology of infection. At the cow level, the proportion of cows with bacteriological cure was 35% and 34% for cows assigned to NC and to CB|CMT-pos, respectively [OR_{NC} (95% CI) = 0.9 (0.5, 1.6); P = 0.7284] (Table 4.3). Covariates that remained in the cow-level model because of confounding of the treatment program effect on bacteriological cure risk were the cow lactation number and number of quarter(s) from the same cow from which bacteria was isolated at the laboratory. Among quarters from cows assigned to CB|CMT-pos, the bacteriological cure risk was 65% for CMT-negative quarters from cows that received IMM antibiotic treatment, 54% for CMT-positive quarters from cows that received IMM antibiotic treatment, 48% for CMT-negative quarters from cows that did not receive IMM antibiotic treatment, and 36% for CMT-positive quarters from cows that did not receive

IMM antibiotic treatment. The bacteriological cure risk for GN quarters of cows assigned to NC, CMTB and CB|CMT-pos was 81%, 92% and 82%, respectively. The bacteriological cure risk for GP quarters of cows assigned to NC, CMTB and CB|CMT-pos was 39%, 58% and 49%, respectively.

New IMI Risk

The proportion of quarters with a new IMI was 45% and 44% for quarters from cows assigned to NC and to CMTB, respectively. There was no significant difference in risk for new IMI between quarters from cows assigned to CMTB and quarters from cows assigned to NC [OR_{NC} (95% CI) = 1.0 (0.8, 1.3); P = 0.9919] (Table 4). Covariates that remained in the quarter-level model because of confounding of the treatment program effect on new IMI risk included the quarter CMT result, number of quarter(s) from the same cow from which bacteria was isolated at the laboratory and etiology of infection. At the cow level, the new infection risk was 77% and 82% for cows assigned to NC and to CMTB, respectively [OR_{NC} (95% CI) = 1.2 (0.5, 2.6); P = 0.6479] (Table 4.4). Covariates that remained in the cow-level model because of confounding of the treatment program effect on new IMI risk included the number of quarter(s) from the same cow from which bacteria was isolated at the laboratory and etiology of infection. Among quarters from cows assigned to CMTB, the new IMI risk for quarters that received IMM antibiotic treatment (CMT-positive quarters) was 39% and for quarters that that did not receive IMM antibiotic treatment (CMT-negative quarters) was 48%.

The proportion of quarters with a new IMI was 45% and 46% for quarters from cows assigned to NC and to CB|CMT-pos, respectively. There was no significant difference in risk for new IMI between quarters from cows assigned to CB|CMT-pos and quarters from cows assigned to NC [OR_{NC} (95% CI) = 1.0 (0.8, 1.2); P = 0.9756] (Table 4.4).

Covariates that remained in the quarter-level model because of confounding of the treatment program effect on new IMI risk included the cow lactation number, number of quarter(s) from the same cow from which bacteria was isolated at the laboratory and etiology of infection. At the cow level, the new IMI risk was 77% and 82% for cows assigned to NC and to CB|CMT-pos, respectively [OR_{NC} (95% CI) = 0.9 (0.7, 1.2); P = 0.5547] (Table 4.4). The only covariate that remained in the cow-level model because of confounding of the treatment program effect on new infection risk was cow lactation number. Among quarters from cows assigned to CB|CMT-pos, the new IMI risk was 44% for CMT-negative quarters from cows that received IMM antibiotic treatment, 41% for CMT-positive quarters from cows that received IMM antibiotic treatment, 48% for CMT-negative quarters from cows that did not receive IMM antibiotic treatment, and 48% for CMT-positive quarters from cows that did not receive IMM antibiotic treatment. The overall risk for new IMIs for the three study groups for NG, GN and GP quarters was 44%, 43% and 46%, respectively.

ICR Risk

The ICR risk was 59% and 53% for quarters of cows assigned to NC and to CMTB, respectively. There was no significant differences in the ICR risk (represents the presence

of infection risk, CM risk, or removal from herd risk within 21 days after enrollment) between quarters from cows assigned to CMTB and quarters from cows assigned to NC [OR_{NC} (95% CI) = 0.8 (0.6, 1.1); P = 0.2327] (Table 4.5). The only covariate that remained in the quarter-level model because of confounding of the treatment program effect on the ICR risk was number of quarter(s) from the same cow from which bacteria was isolated at the laboratory. At the cow level, the ICR risk was 87% and 85% for cows assigned to NC and to CMTB, respectively [OR_{NC} (95% CI) = 0.9 (0.5, 1.8); P = 0.8978] (Table 4.5). Covariates that remained in the cow-level model because of confounding the treatment program effect on the ICR risk included the cow lactation number and number of quarter(s) from the same cow from which bacteria was isolated at the laboratory and etiology of infection. Among quarters from cows assigned to CMTB, the new IMI risk for those that received IMM antibiotic treatment (CMT-positive quarters) was 56% and for those that that did not receive IMM antibiotic treatment (CMT-negative quarters) was 51%.

The ICR risk was 59% and 58% for quarters of cows assigned to NC and to CB|CMT-pos, respectively. There was no significant differences in the ICR risk (represents the presence of infection risk, CM risk, or removal from herd risk within 21 days after enrollment) between quarters from cows assigned to CB|CMT-pos and quarters from cows assigned to NC [OR_{NC} (95% CI) = 0.8 (0.6, 1.1); P = 0.2366] (Table 4.5). The only covariate that remained in the quarter-level model because of confounding of the treatment program effect on the ICR risk was number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. At the cow level, the ICR risk was

35% and 47% for cows assigned to NC and to CB|CMT-pos, respectively [OR_{NC} (95% CI) = 1.0 (0.5, 2.0); P = 0.9022] (Table 4.5). The only covariate that remained in the cow-level model because of confounding of the treatment program effect on the ICR risk was number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. Among quarters from cows assigned to CB|CMT-pos, the ICR risk was 52% for CMT-negative quarters from cows that received IMM antibiotic treatment, 58% for CMT-positive quarters from cows that received IMM antibiotic treatment, 59% for CMT-negative quarters from cows that did not receive IMM antibiotic treatment, and 61% for CMT-positive quarters from cows that did not receive IMM antibiotic treatment. The overall ICR risk for the three study groups for NG, GN and GP quarters was 45%, 49% and 74%, respectively.

DISCUSSION

This study evaluated the efficacy of two novel programs designed to identify and treat subclinical mastitis in cows after parturition. These programs differed with NC not only on the administration of IMM antibiotic treatment of quarter IMIs after parturition, but the success of the CMTB and CB|CMT-pos programs also depended upon the accuracy of the CMT and OFC systems. Thus, the CMTB program was based on identifying for treatment cows and quarters based on CMT results alone, and the CB|CMT-pos program was based on the sequential testing using OFC to diagnose Gram-positives in CMT-positive quarters. Another strength of this study was that it measured program effects not

only on the bacteriological cure risk, but also measured program effects on other outcomes that are likely to be of biological, practical and economic importance to dairy producers, including the number of doses of IMM antibiotics used and days that milk was withheld from the market. Moreover, long-term effects of the programs evaluated, including effects on SCC, milk production, clinical mastitis risk, and cull risk throughout the entire lactation, will be reported in a separate manuscript.

All cows assigned to CMTB received IMM antibiotic treatment. Because 38% of cows after parturition had at least a CMT-positive quarter, and because 49% of the quarters from CMT-positive cows tested positive to the CMT, this resulted in applying IMM antibiotic treatment to almost half of the quarters from CMT-positive cows. Conversely, only 40% of the cows and 15% of the quarters assigned to CB|CMT-pos received treatment. Consequently, there was an increase on the days of milk withheld from the market for both study groups where IMM antibiotics were used, CMTB and CB|CMT-pos study groups, compared to NC. Cows assigned to CMTB required 12 hours to apply both IMM treatments and at least a 96 hours milk withdraw period afterwards. The end result was 6.3 days out of the tank after parturition for cows assigned to CMTB versus the 1.7 days required for cows assigned to NC. In cows assigned to CB|CMT-pos, only 40% of the cows with CMT-positive quarters that grew GP on OFC were treated with IMM antibiotics. Conversely, treatment was delayed one day while waiting for the OFC results. The end result was 4.4 days out of the tank for cows assigned to CB|CMT-pos, 2.0 days for non treated cows and 7.8 days for treated cows. The increase in expenses resulting from IMM antibiotic treatment and the additional days out of the tank will be

used to evaluate the overall cost-benefit of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition.

In the present study, the bacteriological cure at the quarter and cow levels was higher for CMT-positive cows assigned to CMTB than for cows assigned to NC. Controlled studies evaluating the efficacy of antibiotic therapy for IMI based on CMT testing in early lactation are scarce, and the ones carried out have reported mixed results. Rosenberg evaluated cure rates and SCC when CMT-positive quarters in cows after parturition were treated with IMM Cephapirin Sodium (Rosenberg *et al.*, 2002). It was determined that by four weeks post-calving, quarters treated with Cephapirin Sodium had significantly increased bacteriological cure rates compared with untreated control quarters. However, cure risks by bacteria classification groups were not reported in this study. Conversely, Wallace *et al.* (2004), also randomly assigning cows with CMT-positive quarters to receive either IMM Cephapirin Sodium or no treatment, found that there was no difference in cure rates for IMM antibiotic-treated quarters for major pathogens compared to the untreated controls. However, there was an advantage for cure rates using antibiotics against environmental streptococcal infections. Quarters with streptococci infections were 3.5 times more likely to cure if treated with sodium cephapirin. Contrary to Wallace *et al.* (2004) findings, in the present study the greater numerical differences in bacteriological cure between quarters from treated cows and quarters from untreated cows occurred at bacteria classification groups other than non-agalactiae streptococci, especially for quarters where *Staphylococcus aureus*, coagulase-negative staphylococci or *Bacillus* spp. were isolated. It may be due to that in our study the bacteriological cure

risk for non-agalactiae streptococci in quarters assigned to NC was already very high, 88%. However, much lower bacteriological cure risk, below 40%, was observed in non treated quarters where *Staphylococcus aureus*, coagulase-negative staphylococci or *Bacillus* spp. were isolated.

The finding that the bacteriological cure risk was higher for CMT-positive cows assigned to CMTB (CMT-positive quarters received IMM antibiotic treatment) than for CMT-positive cows assigned to NC (CMT-positive quarters did not receive IMM antibiotic treatment) (59 vs. 42%), that the bacteriological cure risk was the same for CMT-positive and CMT-negative quarters from CMT-positive cows assigned to NC (42%), and that the bacteriological cure risk was similar (61 vs. 57%) for CMT-negative quarters from CMT-positive cows (did not receive IMM antibiotic treatment) and for CMT-positive quarters from CMT-positive cows (received IMM antibiotic treatment) assigned to CMTB, may suggest that IMM treatment is also efficacious in non treated quarters if any other quarter from the same cows is treated. This finding holds even if the bacteriological cure risk is stratified by etiology of infection. Although variables that we did not take into account could be potential confounders, different etiology of infection between CMT-positive and CMT-negative quarters of CMT-positive cows is not confounding this finding.

Accordingly, it has been reported the transference of antibiotics from treated to non treated quarters (Blobel, 1960; Hawkins et al., 1962; Leonard et al., 1988). This hypothesis requires further study.

It has been suggested that a rapid and accurate method for confirming presence and etiology of infection in CMT-positive quarters is needed, because the CMT test alone lacks specificity in fresh cows and because only Gram-positive pathogens are considered appropriate for antibiotic therapy (Wallace *et al.*, 2004). On-farm culture was used in CMT-positive quarters from CMT-positive cows assigned to CB|CMT-pos to diagnose and guide selective treatment of GP IMI in cows after parturition. The bacteriological cure risk for quarters from cows assigned to CB|CMT-pos was numerically higher, but not significantly different than for quarters from cows assigned to NC. The smaller magnitude of the treatment effect in bacteriological cure risk for quarters assigned to CB|CMT-pos in comparison to quarters assigned to CMTB may be due to random chance, to the imperfect accuracy of the OFC to identify GP, or to the beneficial effect of treatment in GN and/or NG quarters. Lago *et al.* (2009) reported that if the treatment decision of CMT-positive quarters after parturition was based on GP growth on OFC, 26% of the non treated quarters should have received treatment. This low negative predictive value was due to the low sensitivity of the OFC to identify GP when the prevalence on infection was low. Therefore, this quarter bacteriological status misclassification that deprives from IMM treatment CMT-positive quarters that would benefit from it may be biasing towards the null the treatment effect. The withholding of antibiotic treatment from GN quarters does not appear to influence the overall bacteriological cure risk for quarters of cows assigned to CB|CMT-pos since they only represent 4% of all the quarter bacteriological infections. However, it should be remembered that the antibiotic used in the current study was not labeled for use in gram-negative IMI. Future studies should investigate if use of a more broad-spectrum

antibiotic might produce different results in quarters with GN infections. A total of 241 and 244 cows with 942 and 957 quarters were enrolled into each the NC and CB|CMT-pos study groups, respectively. However, because bacteriological cure risk was only calculated from quarters where bacteria were isolated at enrollment, the power to detect a difference in bacteriological cure risk of 8% in excess of 95% confidence and accounting for the clustering of quarters within cows and cows within herds was only 42%.

The ICR risk at 14 and 21-d after enrollment at the quarter and cow levels was numerically higher, although not significant different, for CMT-positive cows assigned to CMTB than for cows assigned to NC. Lago *et al.* (2009) suggested that the ICR risk may be a better outcome measure of both programs treatment decisions than bacteriological cure risk since it represents both the bacteriological cure risk and new infection risk immediately after enrollment, includes in the analysis those quarters where bacteria were not isolated from the enrollment sample (58% of the quarters). The omission of quarters where bacteria were not isolated from the enrollment sample in the bacteriological analysis could introduce selection bias due to omission of data from the analysis. Thus, it may more truly reflect the success of the intervention in the quarter infection status. The inclusion, in the calculation of the ICR risk, of quarters without a follow-up culture result because of clinical mastitis or removal from the herd was also done in an attempt to reduce potential omission bias. Using the ICR risk as a dependent variable, there were no differences between CMT-positive cows assigned to CB|CMT-pos and for cows assigned to NC for those outcomes from 14 to 21-d after enrollment. The explanations given previously, sensitivity limitations of the OFC to identify GP when the prevalence on

infection is low and not enough statistical power to claim numerical differences as statistically different, for not finding significant differences in bacteriological cure between CMT-positive cows assigned to CB|CMT-pos and for cows assigned to NC would explain the no finding of significant differences in the ICR risk.

The use of the CMT alone, or CMT and an OFC system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition resulted in a higher bacteriological cure risk within 21 days after enrollment. Nevertheless, the implementation of both programs implied an additional use of antibiotics and an increase on the days of milk withheld from the market. Long-term outcomes of the intervention such as lactational clinical mastitis incidence, SCC, milk production, reproductive performance and cow survival will be reported in a different manuscript. Increased revenues and additional expenses resulting from implementing both programs, extra labor cost, CMT and OFC implementation costs, antibiotic expenses and more days of milk withheld from the market will be used to evaluate the overall cost-benefit of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition.

CONCLUSIONS

The use of the CMT to identify cows and quarters for the strategic treatment with IMM Cephapirin Sodium of subclinical IMI after parturition resulted in a significantly higher bacteriological cure risk and reduced the ICR risk within 21 days after enrollment. The implementation of this program required the administration of IMM treatment to 49% of

the quarters from cows with at least a CMT-positive quarter (38% of cows after parturition) and extended the time that milk is withheld from the market from 1.7 to 6.3 days. The selection for treatment of only CMT-positive quarters where GP bacteria was isolated using OFC required less antibiotic use (40% of the cows and 15% of the quarters of the cows with at least a CMT-positive quarter) and days out of the tank (4.4 days), however the higher numerical bacteriological cure risk and lower ICR risk found in comparison to quarters from CMT-positive cows assigned to NC, was not statistically different. Results of this study, in addition to long-term outcomes (to be reported separately), will be used to evaluate the overall cost-benefit of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition.

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TABLES

Table 4. 1. Cow and quarter level descriptors and etiology of infection at enrollment for CMT-positive cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Culture-Based
Sample Size			
# of quarters enrolled	942	917	957
# of cows enrolled	241	232	244
Parity [% (n)] – Cow Level			
1 st	42 (102)	49 (114)	49 (118)
2 nd	29 (69)	25 (57)	21 (52)
3 rd +	29 (70)	26 (61)	30 (74)
DIM at Enrollment (days) – Cow Level			
Mean	1.4 (241)	1.4 (232)	1.4 (244)
Median	1.0 (241)	1.0 (232)	1.0 (244)
CMT Score [% (n)] – Quarter Level			
Negative	46 (430)	49 (440)	50 (471)
1+	35 (324)	31 (282)	28 (261)
2+	13 (117)	16 (145)	14 (130)
3+	6 (59)	4 (40)	9 (84)
Number of Quarters with Bacterial Growth per Cow [% (n)] – Cow Level			
0	26 (57)	22 (45)	23 (49)
1	23 (49)	28 (57)	25 (54)
2	25 (55)	23 (47)	26 (55)
3	14 (30)	19 (37)	17 (36)
4	12 (25)	8 (15)	9 (20)
Etiology [% (n)] – Quarter Level			
No growth	58 (513)	57 (470)	58 (508)
Gram-negatives	2 (19)	2 (16)	2 (15)
<i>Escherichia coli</i>	1 (11)	1 (9)	1 (8)
<i>Klebsiella</i> spp.	<1 (4)	<1 (4)	<1 (5)
Gram-positives	38 (333)	39 (322)	39 (338)
Non-ag. streptococci	4 (39)	6 (50)	5 (40)
<i>Staphylococcus</i> spp.	27 (236)	27 (228)	28 (252)
<i>Staph. aureus</i>	1 (11)	2 (15)	<1 (5)
<i>Bacillus</i> spp.	5 (43)	3 (29)	5 (47)
Other	1 (12)	<1 (4)	<1 (4)
Mix Infection / Contaminated	2 (17)	3 (21)	2 (17)

Table 4. 2. Risk to receive IMM antibiotic therapy for all cows enrolled or only CMT-positive cows, and days out of the tank for cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Treatment Effect		Culture-Based	Treatment Effect	
			Estimate (95% CI)	<i>P</i> -value		Estimate (95% CI)	<i>P</i> -value
Risk to Receive IMM Antibiotic Therapy^c [% (n)] – All Cows Enrolled							
Quarter Level	0 (2474)	19 (2449)			6 (2489)		
Cow Level	0 (630)	37 (621)			15 (633)		
Risk to Receive IMM Antibiotic Therapy^c [% (n)] – CMT-Positive Cows							
Quarter Level	0 (942)	50 (917)			15 (957)		
Cow Level	0 (241)	100 (232)			40 (244)		
Days Out of the Tank [Mean ± SD (n)] – Cow Level							
Treated	---	6.3 ± 2.2 (227)			7.8 ± 3.1 (96)		
Non treated	1.7 ± 1.6 (238)	---			2.0 ± 2.0 (142)		
All Cows	1.7 ± 1.6 (238)	6.3 ± 2.2 (227)	Diff _{NC} = 4.7 (4.4, 5.0)	<0.0001	4.4 ± 3.8 (238)	Diff _{NC} = 2.5 (2.0, 3.0)	<0.0001

Table 4. 3. Quarter level bacteriological cure risk at 14±3 and 21±3 days after enrollment for cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Treatment Effect		Culture-Based	Treatment Effect	
			Estimate (95% CI)	P-value		Estimate (95% CI)	P-value
Bacteriological Cure Risk [% (n)]							
Quarter level	42 (280)	59 (252)	OR _{NC} = 2.4 (1.5, 3.7)	0.0002	50 (260)	OR _{NC} = 1.5 (0.9, 2.4)	0.0782
Cow level	35 (133)	47 (129)	OR _{NC} = 1.6 (0.9, 2.7)	0.1106	34 (132)	OR _{NC} = 0.9 (0.5, 1.6)	0.7284
Bacteriological Cure Risk by Etiology [% (n)] – Quarter Level							
Gram-negatives	81 (16)	92 (12)			82 (11)		
Gram-positives	39 (260)	58 (240)			49 (246)		
Non-ag streptococci	88 (32)	86 (35)			84 (31)		
<i>Staphylococcus</i> spp.	31 (182)	48 (171)			43 (175)		
<i>Staph. aureus</i>	30 (10)	73 (11)			---		
<i>Bacillus</i> spp.	39 (33)	77 (22)			56 (36)		
Bacteriological Cure Risk by CMT Result and IMM Antibiotic Administration [% (n)] – Quarter Level							
CMT-neg quarters							
from treated cows	---	57 (114)			65 (43)		
CMT-pos quarters							
from treated cows	---	61 (138)			54 (90)		
CMT-neg quarters							
from non-treated cows	42 (113)	---			48 (69)		
CMT-pos quarters							
from non-treated cows	42 (167)	---			36 (58)		

Table 4. 4. Quarter level new IMI risk at 14±3 and 21±3 days after enrollment for cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Treatment Effect		Culture-Based	Treatment Effect	
			Estimate (95% CI)	P-value		Estimate (95% CI)	P-value
New IMI Risk [% (n)]							
Quarter level	45 (740)	44 (674)	OR _{NC} = 1.0 (0.8, 1.3)	0.9910	46 (701)	OR _{NC} = 0.9 (0.7, 1.2)	0.5547
Cow level	77 (122)	82 (107)	OR _{NC} = 1.2 (0.5, 2.6)	0.6479	82 (106)	OR _{NC} = 1.4 (0.7, 2.9)	0.3134
New IMI Risk by Etiology [% (n)] – Quarter Level							
No growth	45 (439)	43 (398)			44 (420)		
Gram-negatives	28 (18)	50 (12)			58 (12)		
Gram-positives	46 (279)	45 (264)			48 (266)		
Non-ag streptococci	41 (34)	51 (39)			50 (32)		
<i>Staphylococcus</i> spp.	43 (195)	40 (186)			48 (192)		
<i>Staph. aureus</i>	30 (10)	62 (13)			---		
<i>Bacillus</i> spp.	68 (37)	56 (25)			45 (38)		
New IMI Risk by CMT Result and IMM Antibiotic Administration [% (n)] – Quarter Level							
CMT-neg quarters from treated cows	---	39 (337)			44 (128)		
CMT-pos quarters from treated cows	---	48 (337)			41 (143)		
CMT-neg quarters from non-treated cows	48 (347)	---			48 (229)		
CMT-pos quarters from non-treated cows	42 (392)	---			48 (200)		

Table 4. 5. Quarter level ICR risk at 14±3 and 21±3 days after enrollment for cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Treatment Effect		Culture-Based	Treatment Effect	
			Estimate (95% CI)	P-value		Estimate (95% CI)	P-value
ICR Risk [% (n)]							
Quarter Level	59 (810)	53 (729)	OR _{NC} = 0.8 (0.6, 1.1)	0.2327	58 (748)	OR _{NC} = 0.8 (0.6, 1.1)	0.2366
Cow Level	87 (200)	85 (184)	OR _{NC} = 1.0 (0.5, 1.8)	0.8978	89 (187)	OR _{NC} = 1.0 (0.5, 2.0)	0.9022
ICR Risk by Etiology [% (n)] – Quarter Level							
No growth	46 (453)	43 (404)			46 (432)		
Gram-negatives	39 (18)	50 (12)			67 (12)		
Gram-positives	78 (306)	67 (269)			75 (279)		
Non-ag streptococci	50 (36)	56 (39)			57 (35)		
<i>Staphylococcus</i> spp.	80 (215)	68 (191)			78 (202)		
<i>Staph. aureus</i>	92 (12)	85 (13)			100 (5)		
<i>Bacillus</i> spp.	88 (40)	68 (25)			68 (38)		
ICR Risk by CMT Result and IMM Antibiotic Administration [% (n)] – Quarter Level							
CMT-neg quarters from treated cows	---	51 (346)			52 (132)		
CMT-pos quarters from treated cows	---	56 (351)			58 (149)		
CMT-neg quarters from non-treated cows	59 (361)	---			59 (238)		
CMT-pos quarters from non-treated cows	59 (422)	---			61 (211)		

CHAPTER V

EFFICACY OF TWO PROGRAMS DESIGNED TO DIAGNOSE AND TREAT SUBCLINICAL INTRAMAMMARY INFECTIONS AFTER PARTURITION ON CLINICAL MASTITIS, SOMATIC CELL COUNT, MILK PRODUCTION, REPRODUCTION AND CULLING DURING LACTATION

The objective of this multi-state multi-herd clinical trial was to investigate the efficacy of using the California Mastitis Test (CMT) alone, or the CMT and an on-farm culture system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition. A total of 1,885 cows were enrolled in the study. Of those, a total of 1,168 cows had a negative CMT result on all four quarters, which were not assigned to any treatment group, and 717 cows with at least one CMT-positive quarter which were randomly assigned to either a) a negative-control group (NC), b) a CMT-based treatment group (CMTB), or c) to a culture-based treatment group given a CMT-positive result (CB|CMT-positive). Quarters from cows assigned to NC did not receive IMM antibiotic treatment. CMT-positive quarters from cows assigned to CMTB received immediate on-label intramammary treatment with Cephapirin Sodium. Quarters from cows assigned to the culture-based treatment program were not treated until the results of on-farm culture were determined after 24 hr of incubation. CMT-positive quarters in the culture-based treatment program that showed Gram-positive growth were treated

according to label instructions using intramammary Cephapirin Sodium. Quarters assigned to CB|CMT-positive showed no growth, Gram-negative or a mix infection did not receive intramammary therapy. The present article reports long-term outcomes of the mentioned study. The hazard risk ratio for a clinical mastitis event during lactation was lower for quarters assigned to CMTB [HR_{NC} (95% CI) = 0.6 (0.4, 0.9); $P = 0.04$] and to CB|CMT-positive [HR_{NC} (95% CI) = 0.6 (0.4, 0.9); $P = 0.02$]. The risk and average days after enrollment to a clinical mastitis event in the same quarter was 10% and 124 days, 8% and 114 days and 12% and 124 days for quarters assigned to CMTB, CB|CMT-pos and to NC, respectively. Similarly, the LSCC was lower for cows assigned to CMTB than for cows assigned to NC [$Diff_{NC}$ (95% CI) = -0.31 (-0.61, 0.01); $P = 0.04$]. However, LSCC, although numerically lower, was not significantly lower for cows assigned to CB|CMT-positive [$Diff_{NC}$ (95% CI) = -0.22 (-0.45, 0.08); $P = 0.14$]. The average LSCC was 2.9, 3.1 and 3.3 for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. Using NC as the reference, there was no significant difference in milk production for cows assigned to CMTB [$Diff_{NC}$ (95% CI) = -0.51 kg/day (-1.94, 0.93); $P = 0.48$] and to CB|CMT-positive [$Diff_{NC}$ (95% CI) = -1.1 (-2.51, 0.31); $P = 0.12$]. The average milk production was 35.1, 34.5 and 35.6 kg for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the pregnancy hazard risk ratio for cows assigned to CMTB [HR_{NC} (95% CI) = 1.0 (0.8, 1.2); $P = 0.99$] or to CB|CMT-positive [HR_{NC} (95% CI) = 1.2 (0.9, 1.6); $P = 0.20$]. The risk and average days after enrollment to conception was 76% and 126 days, 75% and 119 days and 73% and 119 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the hazard risk ratio for removal from the herd for cows assigned

to CMTB [HR_{NC} (95% CI) = 0.9 (0.6, 1.2); $P = 0.46$] or to CB|CMT-positive [HR_{NC} (95% CI) = 0.7 (0.5, 1.0); $P = 0.09$]. The risk and average days after parturition to removal from herd was 27% and 124 days, 24% and 144 days and 31% and 121 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

INTRODUCTION

Subclinical mastitis intramammary infection (IMI) at calving is a common occurrence. Reported prevalence of quarter IMI at parturition in North-American studies ranges from 29% to 63% (Fox *et al.*, 1994; Roberson *et al.*, 1994; Kirk *et al.*, 1996; Oliver *et al.*, 1997; Sargeant *et al.*, 2001; Godden *et al.*, 2003; Wallace *et al.*, 2004). A negative relationship between early lactation somatic cell count (SCC) and SCC, milk production and culling hazard during the first lactation has been established in heifers (De Vliegher *et al.*, 2004; De Vliegher *et al.*, 2005a; De Vliegher *et al.*, 2005b). Similarly, elevated SCC in early lactation was also found to increase the probability of clinical mastitis over the first lactation (Rupp and Boichard, 2000). Also, a negative relationship between subclinical mastitis defined and subsequent reproductive performance in Jersey cows was established previously (Schrick *et al.*, 2000). Consequently, there is a need to prevent these infections from occurring, and to develop treatment strategies to diminish the disease impact.

The California mastitis test (CMT), a qualitative measurement of the SCC in milk, is a cowside screening test for detecting subclinical mastitis. Recent studies using the CMT

test in the first week after calving have suggested that there may be potential for its use as a screening tool to identify subclinical IMI in fresh cows (Sargeant *et al.*, 2001; Wallace *et al.*, 2002; Dingwell *et al.*, 2003). However, a high false positive rate was reported. Dingwell *et al.* (2003) reported that if the CMT yielded a negative result, the producer could be 95% certain the quarter was truly uninfected. However, if the test yielded a positive result, there was a 79% chance that it was a false-positive result.

Controlled studies evaluating the efficacy of antibiotic therapy for IMI based on CMT testing in early lactation are scarce, and the ones carried out have reported mixed results. Rosenberg evaluated cure rates and SCC when CMT-positive quarters in cows after parturition were treated with IMM Cephapirin Sodium (Rosenberg *et al.*, 2002). It was determined that by four weeks post-calving, quarters treated with Cephapirin Sodium had significantly increased cure rates and SCC were significantly reduced compared with untreated control quarters. Conversely, Wallace *et al.* (2004), also randomly assigning cows with CMT-positive quarters to receive either IMM Cephapirin Sodium or no treatment, found that there was no difference in cure rates for IMM antibiotic-treated quarters for major pathogens compared to the untreated controls.

It was suggested that a rapid and accurate method for confirming if infection is present in CMT-positive quarters and type of pathogen is needed, since only Gram-positive pathogens are considered appropriate for antibiotic therapy (Wallace *et al.*, 2004). On-farm culture (OFC), a rapid and inexpensive tool, could conceivably be used as a

confirmatory test, in conjunction as a screening tool (such as CMT), to diagnose and guide strategic treatment of subclinical IMI in cows after parturition.

The objective of this study was to investigate the efficacy of using the CMT alone, or CMT and an OFC system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition. Outcomes evaluated included: a) risk of clinical mastitis, b) somatic cell count, c) milk production, d) risk of conception, and e) risk to removal from herd during lactation.

MATERIALS AND METHODS

Study Design

A randomized controlled field trial was conducted between June 2005 and August 2006 in 14 Holstein herds. In each herd cows were enrolled into the study for a period not longer than 6 months. Study herds, 5 in Minnesota, 1 in Wisconsin and 8 in Ontario, were a convenience sample of commercial dairy farms from the North American Great Lakes Region. Selected producers were required to maintain compliance with the study protocols and record keeping, have trained personnel, individual animal identification, treatment facilities, appropriate drug storage capabilities, refrigeration and freezer capacity, participate in a Dairy Herd Improvement Association (DHIA) testing program, and compliance. Herd size ranged from 40 to 4,000 cows (median 356 cows). Herds' housing systems, milk production and SCC are described elsewhere (Lago *et al.*, 2009).

Enrollment Process

All cows in the first three days after calving were eligible for enrollment unless they exhibited signs of clinical mastitis at time of calving, or had fewer than three functional teats. If the cow met the designated inclusion criteria for enrollment, the herdsman aseptically collected a single milk sample from all four quarters and performed the CMT on individual quarters. If the cow failed to meet the designated enrollment criteria because of clinical mastitis, then a single milk sample was still collected from the affected quarter(s).

California Mastitis Test

Herd personnel performed the CMT on individual quarters. Detailed CMT procedures were described somewhere else (Lago *et al.*, 2009). The interpretation of the CMT results were as follows: a) negative when there was no gel formation; b) 1+ when there was light or mild thickening (includes trace); c) 2+ when moderately thick gel formed; and d) 3+ when a very thick gel was formed (center became elevated like a fried egg). CMT results for each one of the all four quarters were recorded.

Allocation to Treatment Group

For cows with a negative CMT result on all four quarters no treatment group was assigned and no further action was taken with the cow. Previously collected quarter milk samples were frozen. Cows with at least one CMT-positive quarter were randomly assigned following a simple randomization schedule to either a negative-control group (NC), a CMT-based treatment group (CMTB), or to a culture-based treatment group given a CMT-positive result (CB|CMT-positive) by opening a pre-identified envelope following a sequential order.

Treatment Groups

Negative Control Group

Quarter milk samples that had been collected from all quarters from the cow were frozen on-farm at -20 °C. Quarters from cows assigned to the NC group did not receive IMM antibiotic treatment.

CMT-Based Treatment Group

Immediately after enrollment the quarter milk samples that had been collected from all quarters from the cow were frozen on-farm at -20 °C. CMT-positive were then infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). The treatment was repeated once, 12 hours after the first treatment according to label directions. A milk-withdrawal period of at least 96 hours and

a slaughter withdrawal period of four days were followed after the last treatment.

Culture-Based given a CMT-Positive Result Treatment Group

The aseptically collected milk sample(s) from CMT-positive quarter(s) were first cultured on-farm using the Minnesota Easy Culture System (University of Minnesota, St. Paul, MN). This OFC system consists of a bi-plate which is a petri dish with two different types of agar, MacConkey agar on one half selectively grows Gram-negative organisms, while Factor agar on the other half of the plate selectively grows Gram-positive organisms. A sterile cotton swab was dipped into the milk sample and then plated onto the Factor media half of the bi-plate, redipped into the milk, and then applied to the MacConkey media half of the bi-plate. The plate was placed in an on-farm incubator and incubated at approximately 37°C for 24 hours. The quarter milk sample that had been collected was then frozen on-farm at -20 °C. The next day the plate was read and interpreted according to guidelines provided for the Minnesota Easy Culture System. If bacteria did not grow, the plate was returned to the incubator and re-read approximately 24 hours later. Final results for each sample plate were recorded as a) Gram-positive (GP), when bacteria grew only in the Factor agar media of the bi-plate, b) Gram-negative (GN), when bacteria grew only in the MacConkey agar media of the bi-plate, c) No growth (NG), when bacteria did not grow in either media, and d) mix infection when bacteria grew in both media. The decision about initiation of IMM antibiotic therapy the day after enrollment was based on the on-farm culture results. Quarters from which GP bacteria were isolated received the same IMM antibiotic treatment following the same

procedures than quarters assigned to CMTB. If the on-farm culture result was GN, NG or a mix infection, then the quarter did not receive IMM therapy.

Data Analysis – Definition of Outcome Variables

Risk and Days to a Clinical Mastitis Event

Cow ID, affected quarter(s), and date of clinical mastitis cases occurring during lactation were retrieved from dairy farm management or study records. Cows were followed until the end of the current lactation or 12 months after parturition (whichever came first).

Somatic Cell Count and Milk Production

Monthly DHIA SCC and milk production records from individual cows were retrieved for the entire lactation from the on an-farm record keeping system (DairyComp305; Valley Agricultural Software, Tulare, CA). The test records used in this analysis were those up to 12 months after parturition. Milk SCC were log transformed to normalize the data to linear SCC (LSCC) using the linear SCC formula: $LSCC = [\text{LOGe}(\text{SCC})/0.6931] - 3.6439$ (Ali and Shook, 1980).

Risk and Days to Conception

The date of the conception was retrieved from the on an-farm record keeping system (DairyComp305; Valley Agricultural Software, Tulare, CA). For cows with more than

one conception recorded (e.g. due to embryo loss/abortion), the 1st conception date was used (after examination of the individual records for validation). Cows were followed until the end of the current lactation or up to 12 months after parturition (whichever came first).

Risk and Days to Culling

For all cows in the study, the removal date (culling/death) was retrieved from the on-farm record keeping system (DairyComp305; Valley Agricultural Software, Tulare, CA). Cows were followed until the end of the current lactation or up to 12 months after parturition (whichever came first).

Statistical Analysis - Models and Modeling Strategy

Database summaries and plots were used for exploratory data analysis. Basic diagnostics techniques were used to evaluate normality, independence, homoscedasticity, collinearity and linearity of variables. Different models were used to evaluate the CMTB and CB|CMT-positive treatment programs effect on the outcomes of interest using records from cows assigned the NC group as the reference.

General Linear Mixed Models (GLMM) for Continuous Outcome Variables

Continuous outcome variables such as milk yield and LSCC were modeled as a function of explanatory variables using linear multivariable regression. A multilevel GLMM was constructed with milk yield / LSCC as a continuous, normally distributed response variable. The model was specified with random variation allowed in three hierarchical levels; repeated measure of milk yield / LSCC within cow, variation among cows within the herd, and variation among herds. This was accomplished with the MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) by specifying a correlation structure among the repeated measurements of the same cow, and including a random statement to account for clustering of cows within herds. In order to select the most appropriate covariance structure we started with a full model with all confounding covariates already taken into account. Summary statistics and exploratory data analysis plots to explore the covariance structure were created, and one model for each covariance structure was fitted. The correlation structures that were evaluated include simple (no correlation), compound symmetry, banded diagonal, autoregressive, and unstructured (estimating a correlation for each separate correlation). The different correlation structures were evaluated using goodness of fit measures. The goodness of fit measures will include $-2 * \log \text{likelihood}$ ($-2LL$), Akaike's information criterion (AIC), and Bayesian information criterion (BIC).

Covariates such as cow parity, number and the number of quarter(s) from the same cow from which bacteria was isolated at the laboratory and etiology of infection were included in the model if it was a potential confounding variable. To determine if a covariate confounded the treatment effect on the outcome (milk yield or LSCC), the crude estimate of treatment group (CMTB vs. NC) and (CB|CMT-positive vs. NC) was

compared with the adjusted estimate for that third confounding variable. It was concluded that the variable confounded the association between treatment group and outcome variable if the ratio between the difference of the crude estimate and the adjusted estimate versus the crude estimate was greater than 10%. Each variable was examined for potential confounding one at a time by regression. Once the confounder variables were identified, the next step was to place all confounders into a full model with two-way interaction terms between treatment and the confounder. In order to simplify the model each non-significant interaction term was removed one at a time using a backward stepwise approach, starting by the least significant interaction term, and running the model again until there are no non-significant interaction terms in the model. Next, with non-significant interaction terms removed from the model, it was determined whether there were main effect variables in the model that were not in an interaction term that might be a confounder. The least significant term were removed and it was evaluated if this affected the treatment effect estimate, with the goal to assess whether the variable confounded the treatment-outcome relationship. If the variable was an important confounder, it was returned to the model and other variables were assessed one at a time to see if they were confounders. The treatment variable was forced in the model regardless of the P -value. Once all non-significant interaction terms were removed as well as main effect variables that did not confound the exposure-outcome relationship, this was the final model. Final significance was declared at $P < 0.05$.

Time to Event Models

Binary responses with a 'time to event' component such as quarter risk and days to a clinical mastitis event, risk and days to conception, risk and days to removal from herd (culling/death) were modeled using survival analysis. Cox's proportional hazards regression method was used to test the logistic analysis explanatory variables (see previously described covariates) simultaneously for their association with time until event (PROC PHREG). The standard model was extended by including a frailty term reflecting a latent effect associated with each herd and with each cow when the event of interest was at the quarter level.

Cows (and quarters) were censored when the event of interest happened or at the end of a 12 months follow-up period (whichever occurs first). The assumption of independent censoring between both treatment groups was assessed by comparing the proportion of censored cows or quarters between both treatment groups. In addition, a sensitivity analysis looking at situations of complete positive correlation (every cow or quarter censored experienced the event of interest) or negative correlation (censored cows or quarters did not experienced the event of interest) between censoring and the event of interest was done. If the violation of this assumption did not dramatically alter the treatment effect estimate (<10%), it was concluded that censoring did not introduce bias.

RESULTS

Descriptive Data

A total of 1,885 cows were enrolled in the study. Of those, a total of 1,168 cows had a negative CMT result on all four quarters, which were not assigned to any treatment group, and 717 cows with at least one CMT-positive quarter which were randomly assigned to the NC, CMTB or CB|CMT-positive study groups. Two hundred and forty one cows were assigned to NC, 232 cows to CMTB, and 244 cows were assigned to CB|CMT-positive. Cow and quarter level descriptors and etiology of infection at enrollment for the three study groups was described elsewhere (Lago *et al.*, 2009).

Treatment Programs Effects

Risk and Days to Clinical Mastitis

At the quarter level, using quarters from cows assigned to NC as the reference, the risk and days to have a clinical mastitis event after parturition was lower for quarters from cows assigned to CMTB [HR_{NC} (95% CI) = 0.6 (0.4, 0.9); $P = 0.0401$] and to CB|CMT-pos [HR_{NC} (95% CI) = 0.6 (0.4, 0.9); $P = 0.0276$] (Table 4.1 and Figure 4.1). The only covariate that remained in the model because of confounding the CMTB and |CMT-pos treatment programs effects on the risk and days to a clinical mastitis event was the cow lactation number. At the quarter level, the risk and average days after enrollment to a clinical mastitis event in the same quarter was 10% and 124 days, 8% and 114 days and 12% and 124 days for quarters assigned to CMTB, CB|CMT-pos and to NC,

respectively. Clinical mastitis records in one of the 14 herds were not available at the quarter level, but clinical mastitis records were available for all herds at the cow level.

At the cow level, using cows assigned to NC as the reference, the risk and days to have a clinical mastitis event after parturition was not significantly different for cows assigned to CMTB [HR_{NC} (95% CI) = 0.9 (0.6, 1.2); $P = 0.5465$] and to CB|CMT-pos [HR_{NC} (95% CI) = 0.8 (0.6, 1.1); $P = 0.2112$] (Table 5.1 and Figure 5.1). Covariates that remained in the model because of confounding the CMTB and |CMT-pos treatment programs effects on the risk and days to a clinical mastitis event included the cow lactation number and the number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. At the cow level, the risk and average days after enrollment to a clinical mastitis event was 27% and 140 days, 26% and 148 days and 28% and 133 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Somatic Cell Count

Using cows assigned to NC as the reference, the LSCC was lower for cows assigned to CMTB [$Diff_{NC}$ (95% CI) = -0.31 (-0.61, 0.01); $P = 0.0431$] and to CB|CMT-pos [$Diff_{NC}$ (95% CI) = -0.22 (-0.45, 0.08); $P = 0.1442$] (Table 5.1 and Figure 5.2). Other than the variable describing the treatment programs, no other covariates remained in both models. The compound symmetry (CS) correlation structure resulted in the best model fit, based on various goodness-of-fit measures. The average LSCC was 2.9, 3.1 and 3.3 for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Milk Production

Using cows assigned to NC as the reference, there was no significant difference in milk production for cows assigned to CMTB [Diff_{NC} (95% CI) = -0.51 kg/day (-1.94, 0.93); $P = 0.4866$] and to CB|CMT-pos [Diff_{NC} (95% CI) = -1.1 kg/day (-2.51, 0.31); $P = 0.1259$] (Table 5.1 and Figure 5.3). Other than the variable describing the treatment program, no other covariates remained in both models. The compound symmetry (CS) correlation structure resulted in the best model fit, based on various goodness-of-fit measures. The average milk production was 35.1, 34.5 and 35.6 for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Risk and Days to Conception

Using cows assigned to NC as the reference, there was no significant difference in the risk and days to conception for cows assigned to CMTB [HR_{NC} (95% CI) = 1.0 (0.8, 1.2); $P = 0.9942$] or to CB|CMT-pos [HR_{NC} (95% CI) = 1.2 (0.9, 1.6); $P = 0.2014$] (Table 5.1 and Figure 5.4). Covariates that remained in the model because of confounding the CMTB treatment program effect on the risk and days to conception included the cow lactation number and the number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. The only covariate that remained in the model because of confounding the CB|CMT-pos treatment program effect on the risk and days to conception was the number of quarter(s) from the same cow from which bacteria was

isolated at the laboratory. The risk and average days after enrollment to conception was 76% and 126 days, 75% and 119 days and 73% and 119 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. The risk to conception at first breeding was 36%, 38% and 36% for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Risk and Days to Removal from the Herd

Using cows assigned to NC as the reference, although numerically lower, the risk and days to removal from the herd due to culling or death was not significantly lower for cows assigned to CMTB [HR_{NC} (95% CI) = 0.9 (0.6, 1.2); $P = 0.4697$] or to CB|CMT-pos [HR_{NC} (95% CI) = 0.7 (0.5, 1.0); $P = 0.0981$] (Table 5.1 and Figure 5.5). Covariates that remained in the model because of confounding the CMTB treatment program effect on the risk and days to removal from the herd included the cow lactation number and the number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. The only covariate that remained in the model because of confounding the CB|CMT-pos treatment program effect on the risk and days to removal from the herd was the number of quarter(s) from the same cow from which bacteria were isolated at the laboratory. The risk and average days after parturition to removal from the herd was 27% and 124 days, 24% and 144 days and 31% and 121 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

DISCUSSION

This study evaluated two novel programs to identify and treat subclinical mastitis in cows after parturition. These programs differed with NC not only on the administration of IMM antibiotic treatment of quarter IMIs after parturition, but the success of the CMTB and CB|CMT-pos programs also depend on the accuracy of the CMT and OFC system. Thus, the CMTB program was based on identifying for treatment cows and quarters based on CMT results alone, and the CB|CMT-pos program was based on the sequential testing using OFC to diagnose Gram-positives in CMT-positive quarters. Another strength of this study is the evaluation of long-term outcomes of the intervention. They represent the overall economic impact of the intervention, since subclinically IMIs after parturition result in a higher incidence of clinical mastitis, higher SCC, lower milk production, lower reproductive performance, and higher risk of culling. Consequently, these long term outcomes must be evaluated in order to compare the overall biological and economic impact of treatment interventions of IMI after parturition.

Studies in the United Kingdom investigating the significance of IMI during the non-lactating period highlighted that IMI at, and during the dry and immediate post-calving period, increase the risk of clinical mastitis in the next lactation. They reported that 55.6% of clinical mastitis cases due to *Streptococcus uberis* and 33.3% of cases due to *Streptococcus dysgalactiae* were caused by new infections originally acquired during the dry period (Bradley and Green, 2000; Bradley and Green, 2001; Green *et al.*, 2002). In one study DNA fingerprinting of enterobacterial strains was used (Bradley and Green,

2000). Of all the coliform mastitis events monitored during the first 100 d of lactation, 52.6% resulted from an infection originally acquired during the previous non-lactating period. Similarly, elevated SCC in early lactation was also found to increase the probability of clinical mastitis over the first lactation (Rupp and Boichard, 2000).

The present study is the first study evaluating the efficacy of two programs designed to identify and treat IMIs after parturition on reducing the incidence of clinical mastitis during lactation. The risk to a clinical mastitis event was lower for quarters assigned to CMTB and CB|CMT-pos. However, this difference was not significant when the analysis was done at the cow level. This may be due to the fact that, although both treatment programs were successful in reducing the incidence of mastitis at the quarter level, IMIs leading to clinical mastitis occurred during lactation in other quarters different to the ones infected immediately after parturition.

A negative relationship between early lactation SCC and SCC during the first lactation has been established in heifers (De Vliegher *et al.*, 2004; De Vliegher *et al.*, 2005a; De Vliegher *et al.*, 2005b). The strategy of using antibiotic therapy to treat IMI in the prepartum period is controversial. In a study, prepartum antibiotic-treated heifers had significantly lower SCC than untreated control heifers (Oliver *et al.*, 1992; Oliver *et al.*, 1997; Oliver *et al.*, 2003; Oliver *et al.*, 2004). However, a clinical trial evaluating the efficacy of prepartum IMM antibiotic therapy on subclinical mastitis in heifers during early lactation found that while prepartum IMM antibiotic infusion at 7 or 14 d before expected parturition while reduced the number of heifer IMI postpartum, SCC during the

first 200 d of the first lactation was not affected by treatment (Borm *et al.*, 2006).

Controlled studies evaluating the efficacy of antibiotic therapy for IMI based on CMT testing in early lactation also reported mixed results. Rosenberg evaluated cure rates and SCC when CMT-positive quarters in cows after parturition were treated with IMM Cephapirin Sodium (Rosenberg *et al.*, 2002). It was determined that by four weeks post-calving, quarters treated with Cephapirin Sodium had significantly increased cure rates and SCC were significantly reduced compared with untreated control quarters.

Conversely, Wallace *et al.* (2004), also randomly assigning cows with CMT-positive quarters to receive either IMM Cephapirin Sodium or no treatment, found that there was no effect of treatment on SCC at the three first tests after parturition. An explanation for the significant reduction in SCC in the first study and not in the second is that in the first study SCC was measured at the quarter level, and compared SCC from treated and non treated CMT-positive quarters, while in the second study SCC was measured at the cow level. Measuring SCC at the cow level, instead of comparing treated and non treated quarters, the comparison is done between cows that were treated in one or more quarters with not treated cows. Assuming that treatment is effective, some of the quarters from a cow assigned to receive treatment will not benefit from it (ie. were not infected), and some of the non treated quarters would benefit from treatment. Consequently, antibiotic treatment efficacy differences may be biased toward the null because of the composite sample of treated and non treated quarters. In the present study in which both treatment programs were effective in reducing SCC during lactation, SCC was also measured at the cow level. However, the sample size of the study, and consequently the study power or ability to declare differences significant when they exist, was greater.

Somatic cell count in early lactation in heifers has been associated with a decrease in lactation milk production and increased culling (De Vliegher *et al.*, 2005a). Similarly, the relationship between subclinical mastitis defined by milk culture during early lactation and subsequent reproductive performance in Jersey cows was evaluated previously (Schrick *et al.*, 2000). Cows with subclinical mastitis before first insemination had increased days to first breeding, increased days open, and increased services per conception as compared with controls. Again, studies evaluating the IMM antibiotic treatment of heifers before parturition differed on the treatment effect on milk production, reproductive performance and culling. In some studies, prepartum antibiotic-treated heifers produced more milk than untreated control heifers (Oliver *et al.*, 1992; Oliver *et al.*, 1997; Oliver *et al.*, 2003; Oliver *et al.*, 2004). However, a more recent multi-state, multi-herd study evaluating the efficacy of prepartum IMM antibiotic therapy on subclinical mastitis in heifers during early lactation found that milk production and reproductive performance during the first 200 d of the first lactation was not affected by treatment (Borm *et al.*, 2006). In the current study treatment of CMT-positive quarters or only CMT-positive quarters with a Gram-positive result on OFC did not result in a higher milk production. It may be that even if both treatment programs are effective in reducing IMIs after parturition (Lago *et al.*, 2009), the damage already done is permanent and the secretion capacity of subclinically infected mammary glands is not recovered. Similarly, neither one of the programs evaluated in the current study resulted in a higher conception risk at first insemination, nor affected the overall risk and days to conception. The risk for a cow to be removed from the herd, though not significantly different, it was numerically

lower for both intervention groups. This may be attributed to the reduction in clinical mastitis and SCC experienced by cows assigned to both intervention programs.

It has been reported in an accompanying manuscript that the use of the CMT to identify cows and quarters for the strategic treatment with IMM Cephapirin Sodium of subclinical IMI after parturition resulted in a higher bacteriological cure risk within 21 days after enrollment in comparison to quarters from CMT-positive cows assigned to NC. The implementation of this program required the administration of IMM treatment to 49% of the quarters from cows with at least a CMT-positive quarter (38% of cows after parturition) and extended the time that milk is withheld from the market from 1.7 to 6.3 days (Lago *et al.*, 2009). The selection for treatment of only CMT-positive quarters where Gram-positive bacteria was isolated using OFC required less antibiotic use (15% of the quarters from cows with at least a CMT-positive quarter) and days out of the tank (4.4 days). However, the higher numerical bacteriological cure risk found, was not statistically different. Additionally, long-term outcomes of the intervention were evaluated in this manuscript since they represent the overall economic impact of the intervention. In this study, both treatment programs were effective on direct udder health outcomes, clinical mastitis and LSCC. Again, the SCC for cows assigned to CB|CMT-pos was numerically lower than for cows assigned to NC, but not significantly different. Increased incomes from the implementation of both programs, fewer clinical mastitis cases and lower LSCC, in addition to the additional expenses resulting from implementing both programs, extra labor cost, CMT and OFC implementation costs, antibiotic expenses and more days of milk withheld from the market will be used to

evaluate the overall cost-benefit of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition. Moreover, the intervention has direct repercussions in animal welfare by improving udder health and reducing animal suffering due to less clinical mastitis during lactation.

CONCLUSIONS

The treatment with IMM Cephapirin Sodium of cows and quarters based on CMT results alone, or sequential testing using OFC to diagnose Gram-positives in CMT-positive quarters resulted in a significantly lower clinical mastitis risk for both treatment programs, and significantly lower milk SCC during lactation for cows assigned to CMTB. The lactational SCC for cows assigned to CB|CMT-pos was numerically lower than for cows assigned to NC, but not significantly different. However, the implementation of both treatment programs did not result in higher milk production, improved reproductive performance or lower risk for removal from the herd. Increased revenues and the additional expenses resulting from implementing both programs will be used to evaluate the overall cost-benefit of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition.

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TABLES

Table 5. 1. Lactation clinical mastitis events, somatic cell count, daily milk yield and culling for cows assigned to the three study groups.

Parameter	Negative-Control	CMT-Based	Treatment Effect		Culture-Based	Treatment Effect	
			Estimate (95% CI)	<i>P</i> -value		Estimate (95% CI)	<i>P</i> -value
Clinical Mastitis [% (n)]							
Quarter level	12 (899)	10 (870)	HR _{NC} = 0.6 (0.4, 0.9)	0.0401	8 (894)	HR _{NC} = 0.6 (0.4, 0.9)	0.0276
Cow level	28 (241)	27 (232)	HR _{NC} = 0.9 (0.6, 1.2)	0.5465	26 (244)	HR _{NC} = 0.8 (0.6, 1.1)	0.2112
Linear Somatic Cell Count [Mean ± SD (n)]							
Cow level	3.27 (216)	2.96 (215)	Diff _{NC} = -0.3 (-0.6, -0.1)	0.0431	3.05 (226)	Diff _{NC} = -0.2 (-0.5, 0.1)	0.1442
Daily Milk Yield [Mean ± SD (n)]							
Cow level	35.6 (216)	35.1 (215)	Diff _{NC} = -0.5 (-1.9, 0.9)	0.4866	34.5 (226)	Diff _{NC} = -1.1 (-2.5, 0.3)	0.1259
Conception [% (n)]							
Cow level	73 (233)	76 (223)	HR _{NC} = 1.0 (0.8, 1.2)	0.9942	75 (230)	HR _{NC} = 1.2 (0.9, 1.6)	0.2014
Culling / Death [% (n)]							
Cow level	31 (241)	27 (232)	HR _{NC} = 0.9 (0.6, 1.2)	0.4697	24 (244)	HR _{NC} = 0.7 (0.5, 1.0)	0.0981

Figure 5. 1. Kaplan-Meier survival graph representing the probability of a clinical mastitis event during lactation at a given days after parturition (up to 365 days) for quarters assigned to the three study groups. Quarters assigned to the negative-control group are represented by a solid line, quarters assigned to the CMT-based treatment program are represented by a dashed line, and quarters assigned to the culture-based treatment program are represented by a dotted line.

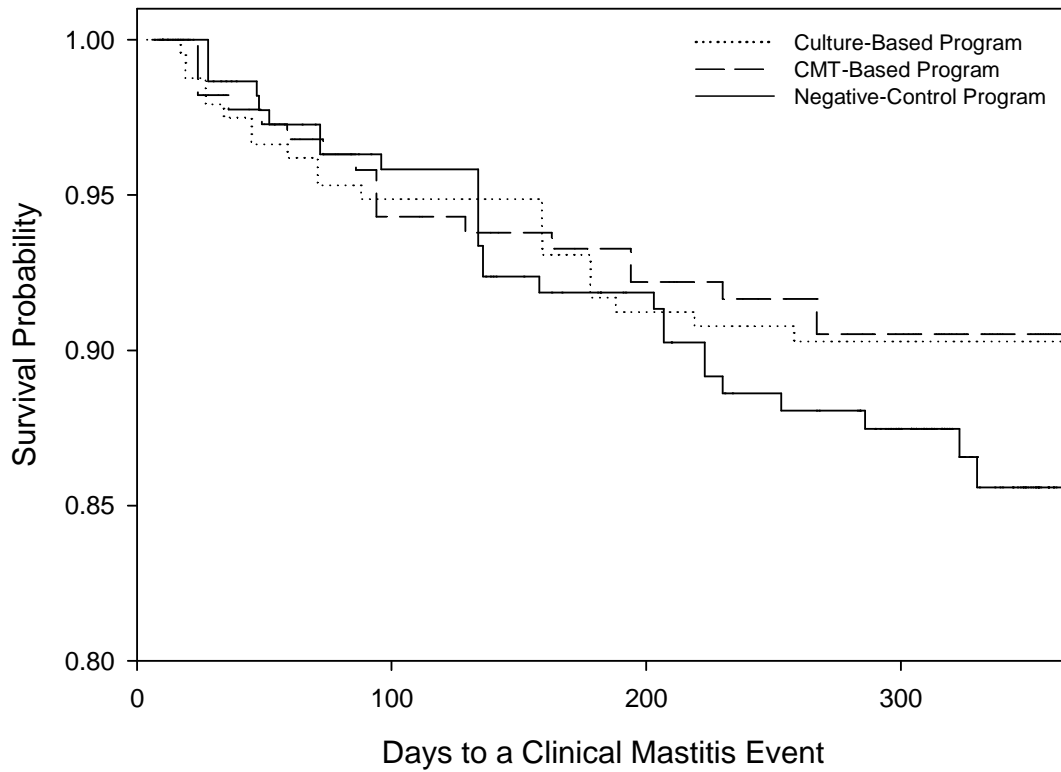


Figure 5. 2. Least square LSCC mean and standard errors during lactation (up to twelve DHIA tests after parturition) for cows assigned to the three study groups. Cows assigned to the negative-control group are represented by a solid line, quarters assigned to the CMT-based treatment program are represented by a dashed line, and quarters assigned to the culture-based treatment program are represented by a dotted line.

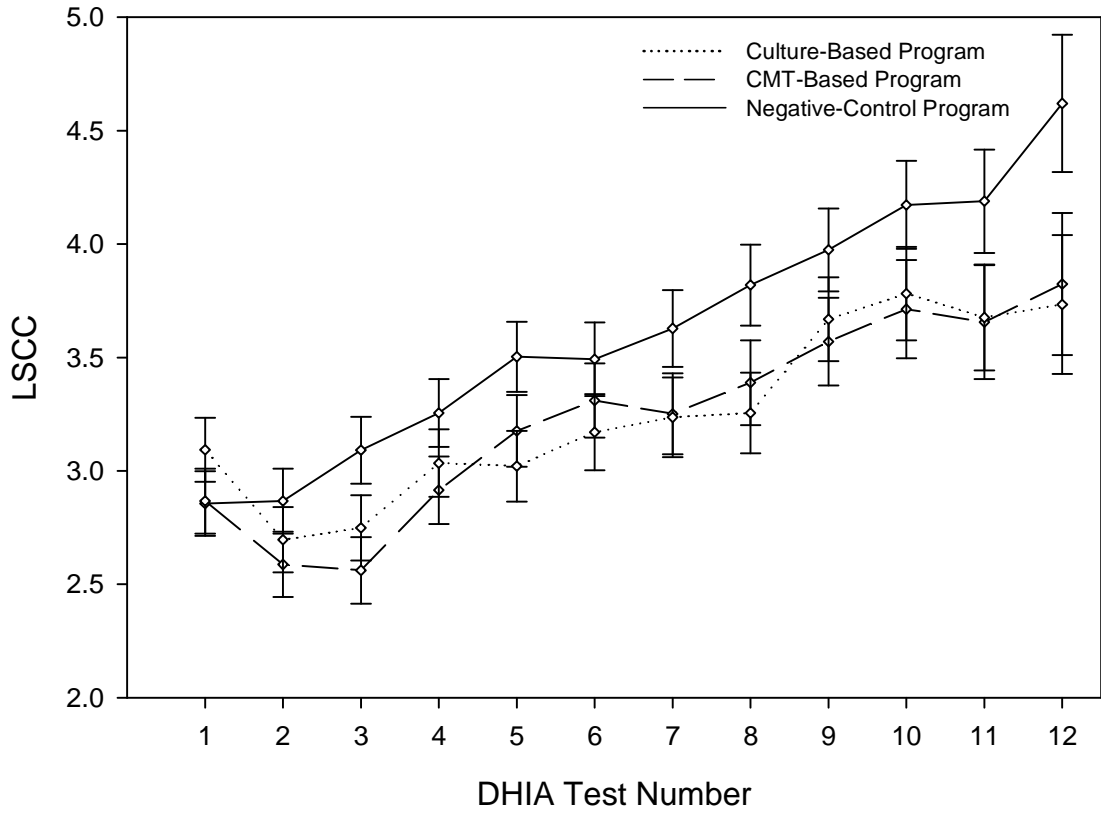


Figure 5. 3. Least square milk yield mean and standard errors during lactation (up to twelve DHIA tests after parturition) for cows assigned to the three study groups. Cows assigned to the negative-control group are represented by a solid line, quarters assigned to the CMT-based treatment program are represented by a dashed line, and quarters assigned to the culture-based treatment program are represented by a dotted line.

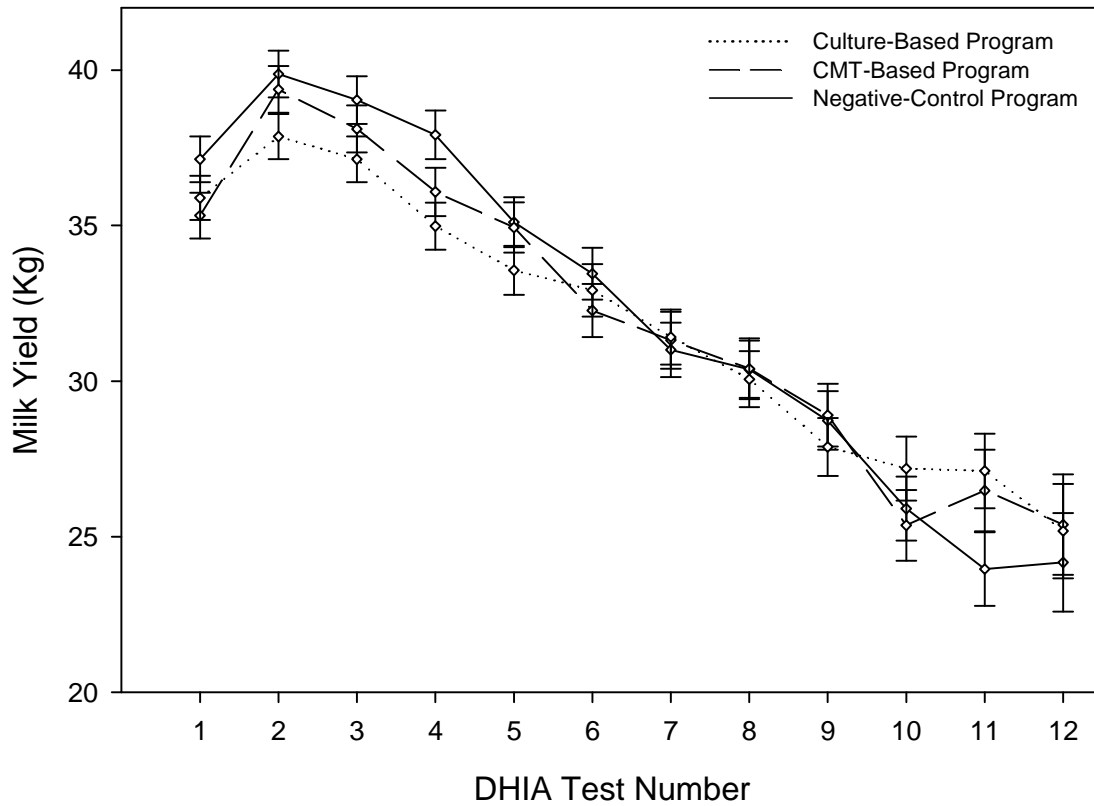


Figure 5. 4. Kaplan-Meier survival graph representing the probability of conception during lactation at a given days after parturition (up to 365 days) for cows assigned to the three study groups. Cows assigned to the negative-control group are represented by a solid line, quarters assigned to the CMT-based treatment program are represented by a dashed line, and quarters assigned to the culture-based treatment program are represented by a dotted line.

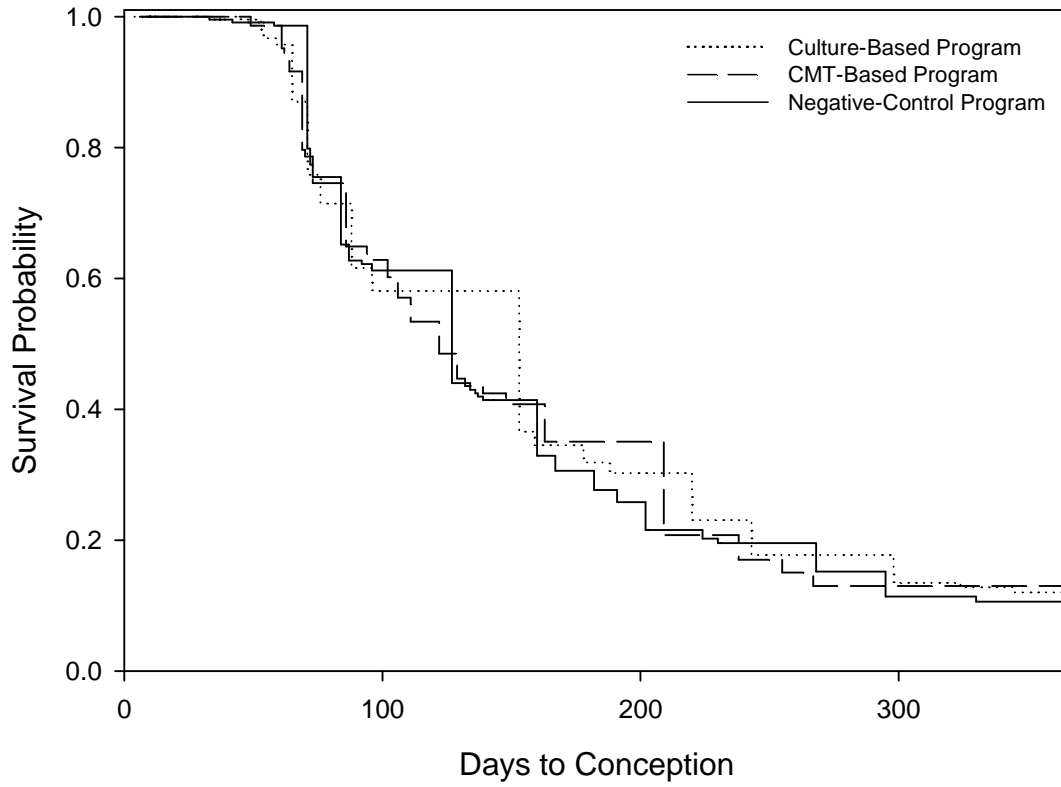
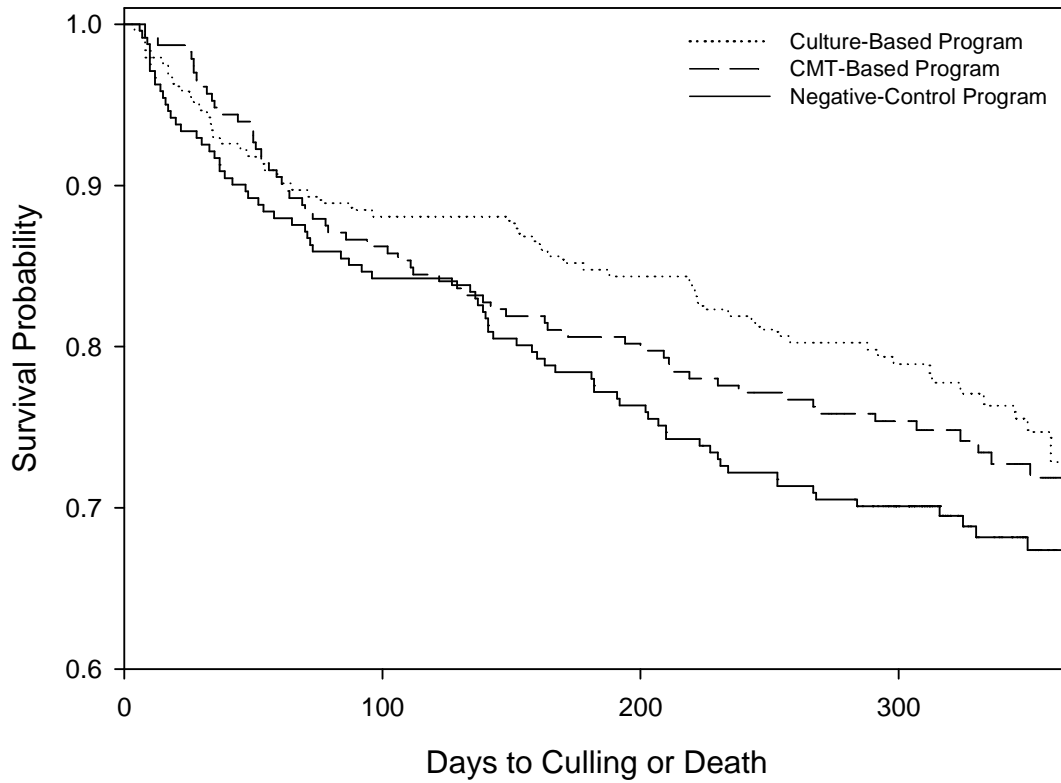


Figure 5. 5. Kaplan-Meier survival graph representing the probability of culling or death during lactation at a given days after parturition (up to 365 days) for cows assigned to the three study groups. Cows assigned to the negative-control group are represented by a solid line, quarters assigned to the CMT-based treatment program are represented by a dash line, and quarters assigned to the culture-based treatment program are represented by a dot line.



CHAPTER VI

VALIDATION OF AN ON-FARM CULTURE SYSTEM TO CORRECTLY IDENTIFY INTRAMAMMARY INFECTIONS IN QUARTER MILK SAMPLES

The first objective of this study was to validate the use of an on-farm milk culture system, the Minnesota Easy Culture Bi-Plate System, to diagnose on farm intramammary infections in quarter milk samples from clinical mastitis cases. The second objective of this study was its validation to diagnose on farm subclinical intramammary infections in CMT-positive quarter milk samples collected after parturition. Agreement beyond chance with laboratory culture results, test characteristics and predictive values were described using three diagnostic interpretations of the bi-plate culture results: a) identification of Gram-positive or Gram-negative bacterial growth (*vs.* no growth), b) identification of Gram-positive bacterial growth (*vs.* no growth and Gram-negative growth), and, c) identification of Gram-negative bacterial growth (*vs.* no growth and Gram-positive growth). A total of 193 quarter milk samples from clinical mastitis cases from 8 herds and 430 CMT-positive quarter milk samples from 14 herds were cultured both on farm using the on-farm culture system and at the laboratory using standard bacteriological culture procedures. The agreement beyond chance, Kappa, between both culture methodologies to classify correctly samples as no-growth, Gram-positive, Gram-negative and mix infections was moderate, 51% in clinical mastitis and 44% in CMT-positive

samples. Kappa for the three diagnostic classifications was 44, 61 and 57%, respectively, in clinical mastitis samples; and 45, 44 and 45%, respectively, in CMT-positive samples. The sensitivity of the bi-plate on-farm culture system for the three diagnostic classifications was 87, 78 and 73%, respectively, in clinical mastitis samples; and 64, 58 and 64%, respectively, in CMT-positive samples. The specificity of the bi-plate on-farm culture system for the three diagnostic classifications was 55, 83 and 87%, respectively, in clinical mastitis samples; and 81, 85 and 97%, respectively, in CMT-positive samples. The predictive value of a positive result for the three diagnostic classifications was 79, 74 and 66%, respectively, in clinical mastitis samples; and 75, 74 and 37%, respectively, in CMT-positive samples. The predictive value of a negative result for the three diagnostic classifications was 69, 86 and 90%, respectively, in clinical mastitis samples; and 72, 74 and 99%, respectively, in CMT-positive samples. The Minnesota Easy Culture Bi-Plate System is a useful cow-side test to correctly identify bacterial growth, Gram-positive bacterial growth, or Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases and in CMT-positive quarter milk samples collected after parturition. Treatment decisions based on identification of bacterial growth, or GP bacterial growth specifically, were correct over 73% of the time.

INTRODUCTION

Ideally, clinical mastitis treatment should be based on milk culture results. However, laboratory culture has not been routinely utilized by many dairies because of the time delay between submission of milk samples and reporting of results. Adoption of rapid

on-farm milk culture systems (OFC) could allow producers to make strategic treatment decisions for clinical mastitis cases, based on knowing the pathogen involved. The postponement of treatment for one day, while waiting for culture results, has been shown to have minimal adverse effects for mild and moderately severe clinical mastitis cases (Wagner *et al.*, 2007). Because antimicrobial treatment of cultures yielding no-growth (NG) or Gram-negative growth (GN) may not be indicated, and because studies show that 50 to 80% of clinical mastitis cases to yield NG or GN on culture, the use of on-farm milk culture for the selective treatment of clinical mastitis may represent a tremendous opportunity to reduce antimicrobial use on commercial dairy farms without sacrificing the efficacy of treatment or the long-term health and production potential of the cow (Roberson *et al.*, 2003). Benefits could include reduced economic cost of therapy, reduced risk of antimicrobial residues in milk, and a reduction in the potential risk for development of antimicrobial resistance in mastitis pathogens (Godden *et al.*, 2007).

Recent studies using the California Mastitis Test (CMT) in the first week after calving have suggested that there may be potential for its use as a screening tool to identify subclinical infection in cows after parturition (Sargeant *et al.*, 2001; Wallace *et al.*, 2002; Dingwell *et al.*, 2003). The authors of these studies suggest a need for cow-side confirmatory test procedures that could be used on CMT-positive quarters to verify infection status and to identify specific pathogens in subclinically infected quarters. Wallace *et al.* (2004) concluded that only gram-positive pathogens (GP) benefit from antibiotic therapy, and that blanket antibiotic treatment of all CMT-positive cows is probably not justified in all herds. A rapid and accurate confirmatory test for verifying if

infection is present in CMT-positive quarters, and type of pathogen present, could benefit producers. On-farm culture systems could be useful as such a confirmatory test.

The Minnesota Easy Culture System (University of Minnesota, Saint Paul, MN), a commercial OFC system, offers two different types of selective culture media systems.

The bi-plate OFC system is a plate with two different types of agar: MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. Alternately, the tri-plate OFC system is a plate with three different types of agar. In addition to including MacConkey agar (GN growth) and Factor agar (GP growth), it also includes a section of MTKT agar which is selective for streptococci.

The first objective of this study was to validate the use of the Minnesota Easy Culture Bi-Plate System to diagnose on farm intramammary infections (IMI) in quarter milk samples from clinical mastitis cases. The second objective of this study was its validation to diagnose on farm subclinical IMI in CMT-positive quarter milk samples collected after parturition. Agreement beyond chance with laboratory culture results, test characteristics, likelihood ratios and predictive values were described using three diagnostic interpretations of the bi-plate culture results: a) identification of Gram-positive or Gram-negative bacterial growth (G) (*vs.* no growth), b) identification of Gram-positive bacterial growth (GP) (*vs.* no growth and Gram-negative growth), and, c) identification of Gram-negative bacterial growth (GN) (*vs.* no growth and Gram-positive growth).

MATERIALS AND METHODS

Study Design

Clinical Mastitis Treatment Study in Cows during Lactation

Details of the clinical mastitis trial have been described elsewhere (Lago *et al.*, 2009). Briefly, cows from eight MN, WI and ON herds with mild or moderate cases of clinical mastitis were randomly assigned to either the positive-control group (PC) or to the on-farm-culture-based treatment group, after herd personnel collected secretion samples from affected quarters. In order to be eligible for enrollment cows had to have at least three functional teats and not exhibit any other condition requiring treatment with systemic antibiotics. Quarter cases from cows assigned to PC were infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). Treatment was repeated once, 12 hours after the first treatment. A secretion sample from quarter cases from cows assigned to the culture-based group was first cultured on-farm onto a bi-plate, and then placed in the freezer for later confirmatory testing at the laboratory for udder health. For cows in the on-farm culture-based treatment group, the decision to initiate IMM antibiotic therapy was made the day after enrollment of the clinical mastitis case, and was based on the OFC results. Quarters from which GP bacteria were isolated or had a mix infection received the same IMM antibiotic treatment following the same procedures than cases assigned to PC. If the on-farm culture result was GN or NG, then the quarter did not receive IMM therapy.

Subclinical Mastitis Treatment Study in Cows after Parturition

Details of subclinical mastitis trial in cows after parturition have been presented elsewhere (Lago *et al.*, 2009). Briefly, cows from 14 MN, WI and ON herds were enrolled into the study in the first three days after calving. In order to be eligible for enrollment cows had to have at least three functional teats and not exhibit any other condition requiring treatment with systemic antibiotics. Herd personnel aseptically collected a quarter milk sample from all four quarters and performed the CMT on individual quarters. For cows with a negative CMT result on all four quarters no treatment was assigned. Cows with at least one CMT-positive quarter were randomly assigned to a negative-control group, a positive-control group (PC), or to a culture-based treatment group. Quarters from cows assigned to the negative-control group did not receive IMM antibiotic treatment. CMT-positive quarters from cows assigned to PC were infused with one syringe (200 mg) of Cephapirin Sodium (Cefa-Lak[®], Fort Dodge Animal Health, Fort Dodge, IA). Treatment was repeated once, 12 hours after the first treatment. A milk sample from CMT-positive quarters from cows assigned to culture-based group was first cultured on-farm using a bi-plate, and then placed in the freezer. The decision to initiate IMM antibiotic therapy was made the day after enrollment, based on the OFC results. Quarters from which GP bacteria were isolated received the same IMM antibiotic treatment following the same procedures than cases assigned to PC. If the on-farm culture result was GN or NG or a mix infection, then the quarter did not receive IMM therapy.

Quarter Milk Sampling

If the cow met the designated inclusion criteria for enrollment in either of the two trials, then the herdsman aseptically collected a single quarter milk sample from all affected quarters in the clinical mastitis study, or from all four quarters in cows after parturition in the subclinical mastitis study. After the routine udder preparation for milking, teat ends were scrubbed with an alcohol-soaked gauze and allowed to dry. Wearing clean gloves, the technician then manually striped approximately 10 ml of milk into an individual 20 ml sterile sample collection vial after discard 2-3 squirts of foremilk. Vials were labeled by cow, quarter, and date.

On-Farm Bacteriological Culture (Bi-plate Minnesota Easy Culture System)

The aseptically collected milk sample(s) from quarter(s) of cows assigned to culture-based was first cultured on-farm using the Minnesota Easy Culture Bi-Plate System (University of Minnesota, Saint Paul, MN). This OFC system consists of a bi-plate which is a petri dish with two different types of agar, MacConkey agar on one half selectively grows GN organisms, while Factor agar on the other half of the plate selectively grows GP organisms. A sterile cotton swab was dipped into the milk sample and then plated onto the Factor media half of the bi-plate, redipped into the milk, and then applied to the MacConkey media half of the bi-plate. The plate was placed in an on-farm incubator and incubated at approximately 37°C for 24 hours. The quarter milk sample that had been

collected was then frozen on-farm at -20 °C. The next day the plate was read and interpreted according to guidelines provided for the Minnesota Easy Culture System. If bacteria did not grow, the plate was returned to the incubator and re-read approximately 24 hours later. Final results for each sample plate were recorded as a) GP, when bacteria grew only in the Factor agar media of the bi-plate, b) GN, when bacteria grew only in the MacConkey agar media of the bi-plate, c) NG, when bacteria did not grow in either media, and d) mix infection when bacteria grew in both media. The decision about initiation of IMM antibiotic therapy the day after enrollment of the clinical mastitis case was based on the on-farm culture results. Quarters from which GP bacteria were isolated or had a mix infection received the same IMM antibiotic treatment following the same procedures than cases assigned to PC. If the on-farm culture result was GN or NG, then the quarter did not receive IMM therapy.

Laboratory Bacteriological Culture

Aerobic culture methodologies for frozen milk samples (enrollment day 0, day 14, day 21) collected on farms were standardized among labs at all three participating sites and performed in accordance with the National Mastitis Council guidelines (NMC, 1999). Briefly, individual quarter milk samples were thawed at the lab at room temperature. While still cold, 0.01 ml of milk was plated onto MacConkey agar and Factor agar using sterile calibrated loops. Factor Agar, similar to KLMB agar (Beatty *et al.*, 1985), selects for GP organisms while inhibiting the growth of GN bacteria with antibiotics. Inoculated plates were incubated at 37°C. After incubation for 18 to 24 h, all plates were observed

for microbial growth. Those plates having growth were recorded and specie identification started. All plates were placed in the incubator for an additional 36 to 48 h and reevaluated for microbial growth. Colonies on MacConkey agar plates were presumptively identified based on colony morphology. Colony color was used as a means of determining if the organism on the plate was a lactose-fermenting organism. Isolates were also Gram stained to assist in organism identification. Organism identity was confirmed using the API 20E test (bioMerieux-Vitek, Inc. Hazelwood, MO). Colonies suspected as being staphylococci based on morphology were confirmed as staphylococci based on catalase reaction and microscopic morphology. Organisms suspected of being *Staphylococcus aureus* were confirmed using the tube coagulase reaction. Those organisms that were catalase-positive and coagulase-negative were classified as *Staphylococcus* spp. Catalase-negative streptococci were streaked onto TKT medium, which is selective for *Streptococcus* spp. only, to determine the esculin reaction and presumptive identification prior to organism confirmation using the API Streptococcus identification system (bioMerieux-Vitek, Inc. Hazelwood, MO).

Any number of colonies from a bacteria species isolated in a milk sample was considered an IMI. A quarter was considered infected when one or two bacterial species were isolated from a quarter milk sample. A quarter sample was considered contaminated if three or more bacterial pathogens were isolated. Laboratory culture results classified as Gram-positive included *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus bovis*, *Enterococcus*, *Staphylococcus aureus*, *Coagulase-negative Staphylococci (Staph species)*, *Corynebacterium bovis*,

Arcanobacter pyogenes, *Citrobacter*, and *Proteus* were isolated. Laboratory culture results classified as Gram-negative included *Escherichia coli*, *Klebsiella spp.*, *Enterobacter*, *Serratia* or *Pseudomonas* were isolated.

Study Population Selection

Milk samples from all quarters enrolled into the culture-based group with both OFC and laboratory culture results were used.

Validation of the Bi-Plate Minnesota Easy Culture System

Test characteristics should be prefixed with the term 'relative' to indicate that the calculations were based on biologically related tests (Minnesota Easy Culture Bi-Plate System and laboratory milk culture media) (Dohoo *et al.*, 2003) with laboratory milk culture being the accepted reference method.

Agreement between the Bi-Plate OFC and Laboratory Culture Results

Agreement between the OFC and laboratory to classify milk sample culture results into NG, GP growth, GN growth and contaminated sample categories was calculated using the Cohen's Kappa statistic. Before assessing kappa, it was assessed whether there was test bias (Dohoo *et al.*, 2003). This was indicated by the proportion classified by both

culture systems in each category (ie $p_1 \neq p_2$, where p_1 and p_2 represent the proportion of NG, GP and GN in each culture system).

Kappa and a 95% confidence interval for Kappa was determined using the PROC FREQ with the /AGREE option of SAS version 9.3 (SAS Institute, Cary, NC). The interpretation of kappa was as follows: a) slight agreement (<20%), b) fair agreement (20 to 40%), c) moderate agreement (40 to 60%), d) substantial agreement (60 to 80%), and e) almost perfect agreement (>80%) (Dohoo *et al.*, 2003).

Ability of the Bi-Plate OFC to Identify Correctly Bacterial Growth

Positive and negative results for the OFC system and laboratory culture results were defined for four diagnostic interpretations:

Correctly Identify Gram-Positive or Gram-Negative Bacterial Growth in Quarter Milk Samples

A positive result is the isolation of bacteria (G), and a negative result is absence of bacterial growth (NG).

Correctly Identify Gram-Positive Bacterial Growth in Quarter Milk Samples

A positive result is the isolation Gram-positive bacteria (GP), and a negative result is absence of bacterial growth (NG) or isolation Gram-negative bacteria (GN). In the clinical mastitis treatment study in cows during lactation, a decision was made to treat with IMM antibiotic in clinical mastitis quarters assigned to the culture-based study group not only if GP bacteria were isolated, but also if a quarter milk sample was classified as a mixed infection on farm. A decision was made not to treat with IMM antibiotic if Gram-negative bacteria or no bacteria were isolated using OFC. As such, a positive OFC result resulted in a decision to treat while a negative OFC result resulted in a decision not to treat.

Correctly Identify Gram-Negative Bacterial Growth in Quarter Milk Samples

A positive result is the isolation Gram-negative bacteria (GN), and a negative result is absence of bacteria growth (NG) or isolation Gram-positive bacteria (GP).

Estimations of Sensitivity, Specificity, Likelihood Ratios and Predictive Values

The sensitivity (Se) of the Minnesota Easy Culture Bi-Plate System is the conditional probability of obtaining a positive result on-farm, given a positive result in the laboratory. Sensitivity and a 95% confidence interval were calculated using the PROC FREQ of SAS version 9.3 (SAS Institute, Cary, NC) with a variable representing the laboratory results in the TABLE statement and selecting only for positive OFC results using the WHERE statement. The specificity (Sp) of the Minnesota Easy Culture Bi-Plate System is the

conditional probability of obtaining a negative result on farm, given a negative result in the laboratory. Specificity and a 95% confidence interval were calculated using the PROC FREQ of SAS version 9.3 (SAS Institute, Cary, NC) with a variable representing the laboratory results in the TABLE statement and selecting only for negative OFC results using the WHERE statement. The likelihood ratio is a prevalence-independent, combined measure of sensitivity and specificity that represents the ratio between the odds of the pretest and post-test probability of a result in the laboratory, given that result using OFC (Dohoo *et al.*, 2003). The likelihood ratio of a positive result (LR+) and the likelihood ratio of a negative result (LR-), as well as 95% confidence intervals were calculated: $LR+ = Se / (1-Sp)$ and $LR- = (1-Se) / Sp$.

The Se and Sp represent the probability of a certain test result (positive or negative), given the known infection status of a quarter sample. However, knowing the Se and Sp does not directly measure the confidence the reader has in the accuracy of an individual test result when applied to quarter samples of unknown bacteriological status. The actual number of true and false diagnostics depend on test characteristics, as well as on the prevalence of infection in the tested population (Dohoo *et al.*, 2003). Predictive values indicate the probability that a quarter milk sample is truly infected (or not infected), depending on whether it tests positive or negative using the OFC system on farm. Predictive values can change with different populations of animals tested with the same test, because they depend on the true prevalence of the condition in the population and test characteristics (Se, Sp). The predictive value of a positive test (PV+) is the probability that, given a positive result on farm, the laboratory milk sample culture result

is also positive. The predictive value of a positive test and a 95% confidence interval were calculated using the PROC FREQ of SAS version 9.3 (SAS Institute, Cary, NC) with a variable representing the OFC results in the TABLE statement and selecting only for positive laboratory results using the WHERE statement. The predictive value of a negative test (PV-) is the probability that, given a negative result on farm, the laboratory milk sample culture result is also negative. The predictive value of a negative test and a 95% confidence interval were calculated using the PROC FREQ of SAS version 9.3 (SAS Institute, Cary, NC) with a variable representing the OFC results in the TABLE statement and selecting only for negative laboratory results using the WHERE statement.

Independent Regression Analysis

Generalized linear mixed models using the SAS GLIMMIX PROC version 9.3 (SAS Institute, Cary, NC), with cow and herd as random effects, were used to estimate Se and Sp by modeling dichotomous variables representing the on farm quarter milk sample culture results (positive or negative) as a function of the laboratory culture results, and vice versa to estimate PV. Covariate information on biological factors collected allows more efficient adjustments for misclassification and may facilitate extrapolation of the results to other populations (Dohoo *et al.*, 2003). Explanatory variables such as cow parity, days in milk (DIM) at clinical mastitis event, and etiology of infection based on laboratory culture results were included in the model if it was a potential confounding variable or if increased the precision of the estimate.

The generalized linear mixed models to estimate Se and Sp were specified as follows:

$\text{logit}(Y_{ijk}) = \alpha + \beta'_{1ij}X_{1ij} + \beta'_{2j}X_{2j} + v_{\text{herd}(i)} + u_{\text{cow}(i)} + \varepsilon_i$ where; Y_{ijk} represent the laboratory culture result, and one of the covariates associated with the vector X_{1ij} represents OFC result; Y_{ij} = the fitted probability of the i^{th} quarter, and the j^{th} cow; α = regression intercept; X_{1ij} = vector of covariates associated with quarter i of cow j ; β'_{1ij} = vector of coefficients for X_{1ij} ; X_{2j} = vector of cow-level exposures for cow j ; β'_{2j} = vector of coefficients for X_{2j} ; $v_{\text{herd}(j)}$ = random effect reflecting the herd-level clustering of cows; $u_{\text{cow}(i)}$ = random effect reflecting the cow level clustering of quarters; and ε_i = residual variation between quarters. Test characteristics for a given set of factor values were estimated as follows: $Se = e^{\mu} / (1 + e^{\mu})$ where $\mu = \alpha + \beta'_{1ij}X_{1ij} + \beta'_{2j}X_{2j}$ when $X_{1ij} = 1$ (model based only in positive laboratory culture results); $Sp = 1 - (e^{\mu} / (1 + e^{\mu}))$ where $\mu = \alpha + \beta'_{1ij}X_{1ij} + \beta'_{2j}X_{2j}$ when $X_{1ij} = 0$ (model based only in negative laboratory culture results). The same approach was used to estimate PV but in this case, the outcome is the quarter milk sample laboratory culture result (positive or negative) as a function of the OFC result. $PV+ = e^{\mu} / (1 + e^{\mu})$ where $\mu = \alpha + \beta'_{1ij}X_{1ij} + \beta'_{2j}X_{2j}$ when $X_{1ij} = 1$ (model based only in positive OFC results); $PV- = 1 - (e^{\mu} / (1 + e^{\mu}))$ where $\mu = \alpha + \beta'_{1ij}X_{1ij} + \beta'_{2j}X_{2j}$ when $X_{1ij} = 0$ (model based only in negative OFC results).

RESULTS

Quarter Samples from Clinical Mastitis Cases in Lactating Cows

Sample Description

A total of 193 quarter milk samples were cultured both on farm using an OFC system and at the laboratory using standard bacteriological culture procedures. The OFC classified 52, 76, 56 and 9 samples as NG, GP, GN and mix infection, respectively (Table 6.1). The laboratories classified 65, 74, 50 and 4 samples as NG, GP, GN and mix infection, respectively. Cow and quarter level descriptors and etiology of infection at enrollment for both study groups of the clinical mastitis trial has been described elsewhere (Lago *et al.*, 2009).

Test Characteristics and Predictive Values

Agreement beyond chance between the Bi-Plate OFC and Laboratory Culture Results

The agreement beyond chance between both culture methodologies to correctly classify samples as NG, GP, GN and mixed infection was moderate [Kappa_{PC} (95% CI) = 51 (41, 60)]. This ranged from 45% to 71% for 4 herds with more than 20 milk sample culture results.

Ability of the Bi-Plate OFC to Correctly Identify Bacterial Growth

Correctly Identify Gram-Positive or Gram-Negative Bacterial Growth in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as G and NG was moderate [$\text{Kappa (95\% CI) = 44 (31, 58)}$] (Table 6.2). This ranged from 32% to 58% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was an 87% probability of isolating bacteria using OFC, given a bacterial growth in the laboratory [$\text{Se}_{\text{OFC}} (95\% \text{ CI}) = 87 (82, 93)$] (Table 6.2). This ranged from 78% to 94% for 4 herds with more than 20 milk sample culture results.

Specificity: There was a 55% probability of not isolating bacteria using OFC, given absence of bacterial growth in the laboratory [$\text{Sp}_{\text{OFC}} (95\% \text{ CI}) = 55 (43, 67)$] (Table 6.2). This ranged from 36% to 70% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating bacteria using OFC were increased to 1.9, given a bacterial growth in the laboratory as compared with samples where bacteria were not isolated in the laboratory. [$\text{LR}_{+\text{OFC}} (95\% \text{ CI}) = 1.9 (1.4, 2.8)$] (Table 6.2). It ranged from 1.5 to 2.8 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating bacteria using OFC were reduced to 0.2, given an absence of bacterial growth in the laboratory, as compared with samples where bacteria were isolated in the laboratory. [$\text{LR}_{-\text{OFC}} (95\% \text{ CI}) = 0.2 (0.1, 0.5)$] (Table

6.2). This ranged from 0.1 to 0.4 for 4 herds with more than 20 milk sample culture results,.

Positive Predictive Value: There was an 79% probability that, given the isolation bacteria using OFC, that the laboratory milk sample culture also resulted in bacterial growth [PV_{+OFC} (95% CI) = 79 (72, 85)] (Table 6.3). This ranged from 72% to 89% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was a 69% probability that, given the isolation bacteria using OFC, that the laboratory milk sample culture also resulted in a bacterial growth [PV_{-OFC} (95% CI) = 69 (57, 82)] (Table 6.3). This ranged from 59% to 78% for 4 herds with more than 20 milk sample culture results.

Identify Gram-Positive Bacterial Infection in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as GP was substantial [Kappa (95% CI) = 61 (49, 72)] (Table 6.2). This ranged from 49% to 79% for 4 herds with more than 20 milk sample culture results of the 8 dairy herds enrolled in the study. Similarly, when an IMM antibiotic treatment decision in clinical mastitis quarters assigned to the culture-based study group was made not only if Gram-positive bacteria were isolated, but also if a quarter milk sample was classified as a mixed infection on farm, then the agreement beyond chance between the two culture methodologies to make a decision to treat was substantial

[Kappa (95% CI) = 61 (49, 72)]. This ranged from 56% to 79% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was a 78% probability of isolating GP bacteria using OFC, given the presence of GP bacterial growth in the laboratory [Se_{OFC} (95% CI) = 78 (68, 87)] (Table 6.2). It was 75% to 100% for 4 herds with more than 20 milk sample culture results, of the 8 dairy herds enrolled in the study. Similarly, there was an 82% probability of making a decision to treat using OFC, given the presence of GP bacterial growth or mix infection in the laboratory [Se_{OFC} (95% CI) = 82 (73, 90)]. This was 75% to 83% for 4 herds with more than 20 milk sample culture results.

Specificity: There was an 83% probability of not isolating GP bacteria using OFC, given the absence of GP bacterial growth in the laboratory [Sp_{OFC} (95% CI) = 83 (77, 90)] (Table 6.2). This ranged from 78% to 100% for 4 herds with more than 20 milk sample culture results, of the 8 dairy herds enrolled in the study. Similarly, there was an 80% probability of making a decision not to treat using OFC, given the absence of GP bacterial growth or mixed infection in the laboratory [Sp_{OFC} (95% CI) = 80 (73, 88)]. This ranged from 76% to 100% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating GP bacteria using OFC were increased to 4.7, given GP bacterial growth in the laboratory, as compared with samples where GP bacteria were not isolated in the laboratory. [LR^+_{OFC} (95% CI) = 4.7 (2.9, 8.8)] (Table 1). This ranged from 3.4 to 6.8 for 4 herds with more than 20 milk sample culture results, of

the 8 dairy herds enrolled in the study. Similarly, the odds of making a decision to treat using OFC were increased to 4.1, given GP bacterial growth or mixed infection in the laboratory, as compared with samples where GP bacteria or mixed infection were not isolated in the laboratory. $[LR_{+OFC} (95\% CI) = 4.1 (2.7, 7.0)]$. This ranged from 3.5 to 6.9 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating GP bacteria using OFC were reduced to 0.3, given an absence of GP bacterial growth in the laboratory, as compared with samples where GP bacteria was isolated in the laboratory. $[LR_{-OFC} (95\% CI) = 0.3 (0.1, 0.4)]$ (Table 6.2). This was 0.3 for 4 herds with more than 20 milk sample culture results, of the 8 dairy herds enrolled in the study. Similarly, the odds of not isolating GP bacteria or mixed infection using OFC were reduced to 0.2, given an absence of GP bacterial growth or mixed infection in the laboratory, as compared with samples where GP bacteria or a mix infection were isolated in the laboratory. $[LR_{-OFC} (95\% CI) = 0.2 (0.1, 0.4)]$. This ranged from 0.2 to 0.3 for 4 herds with more than 20 milk sample culture results.

Positive Predictive Value: There was an 74% probability, given the isolation GP bacteria using OFC, that the laboratory milk sample culture also resulted in GP bacterial growth $[PV_{+OFC} (95\% CI) = 74 (64, 84)]$ (Table 6.3). This ranged from 60% to 100% for 4 herds with more than 20 milk sample culture results. Similarly, there was a 73% probability that, given a decision to treat using OFC, the laboratory milk sample culture resulted on a

GP bacterial growth or mixed infection [PV_{+OFC} (95% CI) = 73 (64, 82)]. This ranged from 65% to 100% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was an 86% probability, given the isolation GP bacteria using OFC, that the laboratory milk sample culture also resulted in GP bacterial growth [PV_{-OFC} (95% CI) = 86 (80, 93)] (Table 6.3). This ranged from 76% to 88% for 4 herds with more than 20 milk sample culture results, of the 8 dairy herds enrolled in the study. Similarly, there was an 87% probability that, given a decision not to treat using OFC, that the laboratory milk sample culture did not result in GP bacterial growth or mixed infection [PV_{-OFC} (95% CI) = 87 (81, 93)]. This ranged from 79% to 90% for 4 herds with more than 20 milk sample culture results.

Correctly Identify Gram-Negative Bacterial Infection in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as GN was moderate [$Kappa$ (95% CI) = 57 (44, 70)] (Table 6.2). This ranged from 53% to 73% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was a 73% probability of isolating GN bacteria using OFC, given the presence of GN bacterial growth in the laboratory [Se_{OFC} (95% CI) = 73 (60, 85)] (Table 6.2). This ranged from 60% to 100% for 4 herds with more than 20 milk sample culture results.

Specificity: There was an 87% probability of not isolating GN bacteria using OFC, given the absence of GN bacterial growth in the laboratory [Sp_{OFC} (95% CI) = 87 (81, 92)] (Table 6.2). This ranged from 77% to 92% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating GN bacteria on farm were increased to 5.4, given GN bacterial growth in the laboratory, as compared with samples where GN bacteria were not isolated in the laboratory. [LR_{+OFC} (95% CI) = 5.4 (3.2, 10.9)] (Table 6.2). This ranged from 4.3 to 7.4 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating GN bacteria using OFC were reduced to 0.3, given an absence of GN bacterial growth in the laboratory, as compared with samples where GN bacteria were isolated in the laboratory. [LR_{-OFC} (95% CI) = 0.3 (0.2, 0.5)] (Table 6.2). This ranged from 0 to 0.4 for 4 herds with more than 20 milk sample culture results.

Positive Predictive Value: There was a 66% probability that, given the isolation of GN bacteria using OFC, that the laboratory milk sample culture also resulted in GN bacterial growth [PV_{+OFC} (95% CI) = 66 (54, 78)] (Table 6.3). This ranged from 46% to 75% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was a 90% probability that, given the isolation GN bacteria using OFC, that the laboratory milk sample culture also resulted in GN bacterial growth [$PV_{\text{OFC}} (95\% \text{ CI}) = 90 (85, 95)$] (Table 6.3). This ranged from 85% to 100% for 4 herds with more than 20 milk sample culture results.

Quarter Samples Collected after Parturition from CMT-Positive Quarters

Sample Description

Four hundred and thirty quarter milk samples were cultured both on farm using an OFC system and at the laboratory using standard bacteriological culture procedures. The OFC classified 258, 142, 19 and 11 samples as NG, GP, GN and mix infection, respectively (Table 6.1). The laboratories classified 228, 181, 11 and 10 samples as NG, GP, GN and mixed infection, respectively. Cow and quarter level descriptors and etiology of infection at enrollment for both study groups have been described elsewhere (Lago *et al.*, 2009).

Test Characteristics and Predictive Values

Agreement beyond chance between the Bi-Plate OFC and Laboratory

Culture Results

The agreement beyond chance between both culture methodologies to classify samples as NG, GP, GN and mixed infection was moderate [$Kappa_{pc}$ (95% CI) = 44 (36, 51)]. It ranged from 40% to 49% for 4 herds with more than 20 milk sample culture results.

Ability of the Bi-Plate OFC to Correctly Identify Bacterial Growth

Correctly Identify Gram-Positive or Gram-Negative Bacterial Infection in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as GP was moderate [$Kappa$ (95% CI) = 45 (37, 54)] (Table 6.2). This ranged from 40% to 45% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was a 64% probability of isolating bacteria using OFC, given the presence of bacterial growth in the laboratory [Se_{OFC} (95% CI) = 64 (57, 70)] (Table 6.2). It ranged from 65% to 94% for 4 herds with more than 20 milk sample culture results.

Specificity: There was an 81% probability of not isolating bacteria using OFC, given the absence of bacterial growth in the laboratory [Sp_{OFC} (95% CI) = 81 (76, 86)] (Table 6.2). This ranged from 44% to 85% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating bacteria using OFC were increased to 3.4, given a bacterial growth in the laboratory as compared with samples where bacteria were not isolated in the laboratory. [LR_{+OFC} (95% CI) = 3.4 (2.4, 5.1)] (Table 6.2). This ranged from 1.7 to 3.9 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating bacteria using OFC were reduced to 0.4, given the absence of bacterial growth in the laboratory as compared with samples where bacteria were isolated in the laboratory. [LR_{-OFC} (95% CI) = 0.4 (0.3, 0.6)] (Table 6.2). This ranged from 0.1 to 0.5 for 4 herds with more than 20 milk sample culture results.

Positive Predictive Value: There was a 75% probability, given the isolation of bacteria using OFC, that the laboratory milk sample culture also resulted in bacterial growth [PV_{+OFC} (95% CI) = 75 (69, 81)] (Table 6.3). This ranged from 59% to 85% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was a 72% probability, given the isolation bacteria using OFC, that the laboratory milk sample culture also resulted in bacterial growth [PV_{-OFC} (95% CI) = 72 (66, 77)] (Table 6.3). This ranged from 54% to 85% for 4 herds with more than 20 milk sample culture results.

Correctly Identify Gram-Positive Bacterial Infection in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as GP was moderate [Kappa (95% CI) = 44 (36, 53)] (Table 6.2). This ranged from 36% to 62% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was a 58% probability of isolating GP bacteria using OFC, given the presence of GP bacterial growth in the laboratory [Se_{OFC} (95% CI) = 58 (51, 65)] (Table 6.2). This was 46% to 88% for 4 herds with more than 20 milk sample culture results.

Specificity: There was an 85% probability of not isolating GP bacteria using OFC, given the absence of GP bacterial growth in the laboratory [Sp_{OFC} (95% CI) = 85 (81, 90)] (Table 6.2). This ranged from 65% to 88% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating GP bacteria using OFC were increased to 3.9, given the presence of GP bacterial growth in the laboratory, as compared with samples where bacteria were not isolated in the laboratory. [LR_{+OFC} (95% CI) = 3.9 (2.6, 6.2)] (Table 6.2). This ranged from 2.4 to 4.3 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating GP bacteria using OFC were reduced to 0.5, given the absence of GP bacterial growth in the laboratory, as compared with samples where GP bacteria were isolated in the laboratory. [LR_{-OFC} (95% CI) = 0.5

(0.4, 0.6)] (Table 6.2). This ranged from 0.2 to 0.6 for 4 herds with more than 20 milk sample culture results.

Positive Predictive Value: There was a 74% probability, given the isolation GP bacteria using OFC, that the laboratory milk sample culture also resulted in GP bacterial growth [PV_{+OFC} (95% CI) = 74 (67, 81)] (Table 6.3). This ranged from 58% to 85% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was a 74% probability, given the isolation GP bacteria using OFC, the laboratory milk sample culture also resulted in GP bacterial growth [PV_{-OFC} (95% CI) = 74 (69, 79)] (Table 6.3). This ranged from 69% to 82% for 4 herds with more than 20 milk sample culture results.

Correctly Identify Gram-Negative Bacterial Infection in Quarter Milk Samples

Kappa: The agreement beyond chance between both culture methodologies to classify milk sample culture results as GN was moderate [$Kappa$ (95% CI) = 45 (22, 67)] (Table 6.2). This ranged from 39% to 74% for 4 herds with more than 20 milk sample culture results.

Sensitivity: There was a 64% probability of isolating GN bacteria using OFC, given the presence of GP bacterial growth in the laboratory [Se_{OFC} (95% CI) = 64 (35, 92)] (Table

6.2). This ranged from 60% to 100% for 4 herds with more than 20 milk sample culture results.

Specificity: There was an 87% probability of not isolating GN bacteria using OFC, given the absence of GN bacterial growth in the laboratory [Sp_{OFC} (95% CI) = 97 (96, 99)] (Table 6.2). This ranged from 89% to 99% for 4 herds with more than 20 milk sample culture results.

Positive Likelihood Ratio: The odds of isolating GN bacteria using OFC were increased to 22.3, given the presence of GN bacterial growth in the laboratory as compared with samples where GN bacteria were not isolated in the laboratory. [LR_{+OFC} (95% CI) = 22.3 (7.9, 72.5)] (Table 6.2). This ranged from 0 to 84.7 for 4 herds with more than 20 milk sample culture results.

Negative Likelihood Ratio: The odds of not isolating GN bacteria using OFC were reduced to 0.4, given the absence of GN bacterial growth in the laboratory, as compared with samples where bacteria were isolated in the laboratory. [LR_{-OFC} (95% CI) = 0.4 (0.1, 0.7)] (Table 6.2). This ranged from 0 to 1 for 4 herds with more than 20 milk sample culture results.

Positive Predictive Value: There was a 37% probability, given the isolation of GN bacteria using OFC, the laboratory milk sample culture also resulted in GN bacterial

growth [PV_{+OFC} (95% CI) = 37 (15, 59)] (Table 6.3). This ranged from 25% to 100% for 4 herds with more than 20 milk sample culture results.

Negative Predictive Value: There was a 98% probability, given the isolation GN bacteria using OFC, the laboratory milk sample culture also resulted in GN bacterial growth [PV_{-OFC} (95% CI) = 99 (98, 100)] (Table 6.3). This ranged from 94% to 100% for 4 herds with more than 20 milk sample culture results.

Independent Regression Analysis for Clinical Mastitis and CMT-Positive Quarter Samples

Including explanatory variables such as cow parity, days in milk (DIM) at clinical mastitis event, and etiology of infection based on laboratory culture result (for the Se and Sp estimations), the ratio between the difference of the crude estimate and the adjusted estimate versus the crude estimate was not greater than 10%, therefore, it was concluded that those variables did not confound the test characteristics or predictive values estimates. Models were also compared using goodness of fit measures and the precision of the estimate was not improved by the inclusion of the explanatory variables in the models. Consequently, test characteristics and predictive values estimates obtained from the independent regression analyses are not reported because they do not differ substantially from those reported previously.

Relationship between Test Characteristics on Individual Herds and Herd Prevalence of Infection

In the clinical mastitis study the herd prevalence (the proportion of clinical quarter samples submitted that yielded a positive laboratory culture result) for the different test interpretations ranged from 48 to 77% for G, 31 to 53% for GP, and 17 to 38% for GN in clinical mastitis cases enrolled in the 4 herds with more than 20 milk sample culture results. The width of the probability distribution in herd prevalence (the proportion of CMT-positive quarters from cows after parturition that yielded a positive laboratory culture result) was even more pronounced. Thus, it ranged from 29 to 78% for G, 26 to 64% for GP, and 0 to 13% for GN in quarters enrolled in the 4 herds with more than 20 milk sample culture results.

Though it was not an a priori objective of this study, it was interesting to note that the OFC test characteristics appeared to vary with herd prevalence. For example, as the herd prevalence increased, the Se and Sp of the bi-plate OFC system to identify G increased and decreased, respectively (Figure 6.1). In addition, there was a strong positive correlation between the herd prevalence and the herd proportion of high bacteria count samples (>100 cfu/ml) among samples with bacteria growth ($R^2 = 0.7371$; $P = 0.0369$). The odds for a positive sample to be classified as positive by the Bi-Plate OFC system were 3.5 times higher in samples where high bacteria concentration was identified at the laboratory ($P < 0.0001$). In the 8 herds with more than 20 milk sample culture results of the 22 dairy herds enrolled in both studies, the proportion of high bacteria count samples

(>100 cfu/ml) among samples with bacteria growth ranged from 57 to 70% for the 4 herds where clinical mastitis cases were enrolled, and 48 to 85% of positive result in CMT-positive quarter samples for the 4 herds that enrolled cows after parturition.

DISCUSSION

Withholding of antibiotic treatment for NG cases is a common practice when OFC is used to make clinical mastitis treatment decisions. In a producer survey describing the use of this OFC system on 52 commercial dairy farms, 67% of the producers reported that antibiotic treatment was not administered in NG cases (Neeser et al., 2006). The overall ability of the bi-plate OFC system to identify bacterial growth from clinical mastitis samples on-farm was comparable to the previously reported of when the test was evaluated in a laboratory setting (Hochhalter et al., 2006).

The prevalence of bacterial isolation in clinical mastitis cases enrolled in the current study was 66% according to the laboratory culture reference method. Given this prevalence of bacterial isolation and the test characteristics of the bi-plate OFC system, $Se = 87\%$ and $Sp = 55\%$, the percentage of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) was 58, 19, 15 and 8, respectively (Table 6.3). If the clinical mastitis treatment decision was only based on bacterial growth using OFC, 21% of the treated clinical mastitis cases should not have received treatment and 31% of the non treated clinical mastitis cases should have received treatment. Overall, the correct treatment decision was made for 77% of clinical mastitis quarters.

The prevalence of bacterial isolation in CMT-positive quarters of cows enrolled after parturition was 47% according to the laboratory culture reference method. Given this prevalence of infection and the test characteristics of the bi-plate OFC system, Se = 64% and Sp = 81%, the percentage of TP, TN, FP and FN was 30, 43, 10 and 17, respectively (Table 6.3). If the treatment decision for CMT-positive quarters after parturition was only based on bacterial growth using OFC, 25% of the treated cases should not have received treatment and 28% of the non treated cases should have received treatment. Overall, the correct treatment decision was made for 73 % of CMT-positive quarters after parturition.

In the previously mentioned producer survey, 93% of the producers reported that antibiotic treatment was administered to treat GP cases of clinical mastitis, whereas only 39% and 33% of producers used antimicrobials to treat GN and NG cases, respectively (Neeser et al., 2006). Therefore, the most common use of the bi-plate OFC system may be to identify GP growth. Test characteristics from the current studies were comparable to a previous report in which the test was evaluated in a laboratory setting (Hochhalter et al., 2006). However, higher Se and lower Sp was reported in a different report (McCarron et al., 2008). The ability to identify bacterial growth has also been evaluated using a different selective culture system, the Petrifilm™ system (3M Microbiology, St Paul, MN). These are sample-ready selective culture media that are marketed for rapid bacteriological isolation and enumeration of bacteria from food products. Petrifilm™ products that are potentially useful for diagnosis of mastitis include Petrifilm™ Aerobic count plates, Coliform count plates and Staph Express count plates (Silva et al., 2004). When the Petrifilm™ Aerobic count and Coliform count plates were evaluated to identify

GP bacteria in fresh refrigerated samples at the laboratory, it performed similarly to the Minnesota Easy Culture Bi-Plate System when a cut-point of 5 colonies in the Petrifilm™ Aerobic count plate and 20 colonies in the Coliform count plate (McCarron et al., 2007). In addition to the need of establishing a cut-point for the Petrifilm™ OFC system to perform similarly to the bi-plate OFC system, authors reported that because of artifact created by mastitis debris, samples were subsequently diluted 1:10 with sterile water before being placed on the Petrifilms™. Another commercial OFC system is the Hymast® Bacteriological Test System which is a selective media bacteriological test system for detection of GP and GN organisms in milk. It has been reported that the Hymast® test is useful for determining GP growth when the test is read at 36 hours of incubation. At earlier readings, 12 and 24 hours after incubation, Se was unacceptable low. However, although Se at 36 hours ranged from 80 to 91% among 5 different readers, Sp was poor ranging from 30 to 45%. The Hymast® test is not currently commercially available.

The prevalence of GP bacterial growth in clinical mastitis cases enrolled in the study was 37% according to the laboratory culture reference method. Given this prevalence of infection and the test characteristics of the bi-plate OFC system, Se = 78% and Sp = 83%, the percentage of TP, TN, FP and FN was 29, 52, 11 and 8, respectively (Table 6.3). If the clinical mastitis treatment decision was based on GP growth, 26% of the treated cases should not have received treatment and 14% of the non treated cases should have received treatment. Overall, the correct treatment decision was made for 81% of clinical mastitis quarters. The prevalence of GP bacterial growth in CMT-positive quarters of

cows enrolled after parturition was 42% according to the laboratory reference method. Given this prevalence of infection and the test characteristics of the bi-plate OFC system, $Se = 58\%$ and $Sp = 85\%$, the percentage of TP, TN, FP and FN was 24, 49, 9 and 18, respectively (Table 6.3). If the treatment decision for CMT-positive quarters after parturition was based on the presence of GP growth using OFC, 26% of the treated cases should not have received treatment and 26% of the non treated cases should have received treatment. Overall, the correct treatment decision was made for 73 % of CMT-positive quarters after parturition.

The bi-plate OFC system may be used to identify GN bacterial growth and a different treatment protocol may follow this diagnosis. This application of the bi-plate OFC system has not been evaluated before. The prevalence of GN growth in clinical mastitis cases enrolled in the study was 26% according to the laboratory culture. Given this prevalence of infection and the test characteristics of the bi-plate OFC system, $Se = 73\%$ and $Sp = 87\%$, the percentage of TP, TN, FP and FN was 19, 64, 10 and 7, respectively (Table 6.3). If the different clinical mastitis treatment decision was based on GN growth, then 34% of cases receiving treatment should not have received treatment and 10% of the cases that did not receive treatment should have received treatment. Overall, the correct treatment decision was made for 73% of clinical mastitis quarters. The prevalence of GN growth in CMT-positive quarters of cows enrolled after parturition was 3% according to the laboratory culture. Given this prevalence of infection and the test characteristics of the bi-plate OFC system, $Se = 64\%$ and $Sp = 97\%$, the percentage of TP, TN, FP and FN was 2, 94, 3 and 1, respectively (Table 6.3). If the treatment decision for CMT-positive

quarters after parturition was based on GN growth, 63% of the cases receiving treatment should not receive treatment and 1% of the cases that did not receive treatment should have received treatment. The large misclassification rate of samples where GN bacterial growth was not present is due to the very low prevalence of GN bacterial growth in CMT-positive quarters after parturition. Overall, the correct treatment decision was made for 96% of CMT-positive quarters after parturition.

Test characteristics of binary diagnostic tests are often thought of as being independent of disease prevalence. However, in the current study using an example the use of the bi-plate OFC system to identify bacterial growth Se increased with herd prevalence (proportion of samples submitted that yield a positive laboratory culture result), while Sp decreased (Figure 6.1). Therefore, it appears that part of the variation in test characteristics (Se and Sp) among herds may be at least partially explained by differences in herd prevalence of IMI.

The reasoning that test characteristics of binary diagnostic tests are independent of disease prevalence is justified in situations with a truly dichotomous disease status and a homogeneous probability of diagnostic misclassification within the population of truly positive samples and within the population of truly negative samples. However, and using an example the use of the bi-plate OFC system to identify bacterial growth, in our database the odds for a positive sample to be classified as positive by the Bi-Plate OFC system were 3.5 times higher in samples where high bacteria concentration (>100 cfu/ml) was identified at the laboratory. This provides evidence that the bi-plate OFC system, like

most tests, is not inherently dichotomous. For these tests, the magnitude of diagnostic misclassification depends not only on the magnitude of the measurement error of the underlying trait(s), but also on the distribution of the underlying trait(s) in the population relative to the diagnostic cutpoint (Brenner and Gefeller, 1997). At the herd level, there was a strong positive correlation between the proportion of high bacteria count samples among samples with bacteria growth and the prevalence of positive samples at the laboratory, therefore, diagnostic misclassification and prevalence of positive samples were not independent. Given the relatively small sample size used in this analysis (8 herds; 4 clinical mastitis study herds, 4 CMT-positive post-parturient study herds), these findings should be considered preliminary and should be interpreted with caution. The proposed hypothesis, that a relationship exists between herd prevalence and performance of the OFC system, requires further study.

Other possible explanations for differences in test performance between clinical mastitis and milk samples from CMT-positive quarters may be explained by differences in herd prevalence. There may be other differences among dairies in the adequacy of the implementation bi-plate OFC system and/or interpretation of results, or differences in the management of the milk samples that contribute to the variation in test performance.

In the current study the bi-plate OFC system was validated at operational test parameters. “Operational” in this context means that the test parameters will be further used to update diagnostic decisions. The objective was to establish estimates of characteristics of a diagnostic test that can be used for conditioning the interpretation of test results on

misclassifications (Greiner and Gardner; 2000). The validity of bi-plate OFC performance depends on the consequences of both FN and FP test results multiplied by the number of each diagnostic error in a given situation. If the goal of the bi-plate OFC is to identify quarters that will benefit from antibiotic treatment, then the consequence of a FN result is the lost opportunity to treat, thereby reducing the severity and duration of the infection. In the other hand, the consequence of a FP result is an unnecessary treatment cost. The efficacy and cost-benefit of the bi-plate OFC system to guide strategic treatment decisions of GP cases in cows with mild and moderate clinical mastitis has been reported elsewhere (Lago et al., 2009). Likewise, the efficacy and cost benefit of using the CMT and the Bi-Plate OFC in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition when GP bacteria was isolated, has been reported elsewhere (Lago et al, 2009).

CONCLUSIONS

The Minnesota Easy Culture Bi-Plate System is a useful cow-side test to correctly identify bacterial growth, Gram-positive bacterial growth, or Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases and in CMT-positive quarter milk samples collected after parturition. Treatment decisions based on identification of bacterial growth, or GP bacterial growth specifically, were correct over 73% of the time. The validity of bi-plate OFC performance depends on the consequences of missing a FN and the consequences of classifying a FP as positive, multiplied by the number of each diagnostic error in a given situation. A future paper will quantify these

consequences in an analysis of the biological outcomes (eg. post-enrollment SCC, milk yield, clinical mastitis, conception, and removal from herd risk) for cows enrolled into these two studies.

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TABLES

Table 6. 1. Laboratory and on-farm culture (Minnesota Easy Culture Bi-Plate System) results for quarter secretion samples from clinical mastitis cases during lactation and quarter milk samples from CMT-positive quarters from cows after parturition.

Parameter	No Growth	Gram-Positive	Gram-Negative	Mixed Infection
	% (n)	% (n)	% (n)	% (n)
Laboratory Culture Results				
Clinical Mastitis ^a	34 (193)	38 (193)	26 (193)	2 (193)
CMT-Positive ^b	53 (430)	42 (430)	3 (430)	2 (430)
On-Farm Culture Results				
Clinical Mastitis	27 (193)	39 (193)	29 (193)	5 (193)
CMT-Positive	60 (430)	33 (430)	4 (430)	3 (430)

^a Quarter secretion samples from clinical mastitis cases

^b Quarter milk samples from CMT-positive quarters from cows after parturition

Table 6. 2. Agreement beyond chance, test characteristics and likelihood ratios of the Minnesota Easy Culture Bi-Plate System in order to identify bacterial growth, identify Gram-positive bacterial growth or identify Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases during lactation or in quarter milk samples from CMT-positive quarters from cows after parturition.

Parameter	Agreement (%)	Test Characteristics (%)		Likelihood Ratios (OR)	
	Kappa (95% CI)	Se (95% CI)	Sp (95% CI)	LR+ (95% CI)	LR- (95% CI)
Identify Bacterial Growth					
Clinical Mastitis ^a	44 (31, 58)	87 (82, 93)	55 (43, 67)	1.9 (1.4, 2.8)	67 (55, 80)
CMT-Positive ^b	45 (37, 54)	64 (57, 70)	81 (76, 86)	3.4 (2.4, 5.1)	67 (50, 81)
Identify Gram-Positive Bacterial Growth					
Clinical Mastitis	61 (49, 72)	78 (68, 87)	83 (77, 90)	4.7 (2.9, 8.8)	0.3 (0.1, 0.4)
CMT-Positive	44 (36, 53)	58 (51, 65)	85 (81, 90)	3.9 (2.6, 6.2)	0.5 (0.4, 0.6)
Identify Gram-Negative Bacterial Growth					
Clinical Mastitis	57 (44, 70)	73 (60, 85)	87 (81, 92)	5.4 (3.2, 10.9)	0.3 (0.2, 0.5)
CMT-Positive	45 (22, 67)	64 (35, 92)	97 (96, 99)	22.3 (7.9, 72.5)	0.4 (0.1, 0.7)

^a Quarter secretion samples from clinical mastitis cases

^b Quarter milk samples from CMT-positive quarters from cows after parturition

Table 6. 3. Predictive values and true and false diagnostics of the Minnesota Easy Culture Bi-Plate System in order to identify bacterial growth, identify Gram-positive bacterial growth or identify Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases during lactation or in quarter milk samples from CMT-positive quarters from cows after parturition.

Parameter	Predictive Values (%)		Correct ^a (%)		Incorrect ^b (%)	
	PV+ (95% CI)	PV- (95% CI)	TP	TN	FP	FN
Identify Bacterial Growth						
Clinical Mastitis ^c	79 (72, 85)	69 (57, 82)	58	19	15	8
CMT-Positive ^d	75 (69, 81)	72 (66, 77)	30	43	10	17
Identify Gram-Positive Bacterial Growth						
Clinical Mastitis	74 (64, 84)	86 (80, 93)	29	52	11	8
CMT-Positive	74 (67, 81)	74 (69, 79)	24	49	9	18
Identify Gram-Negative Bacterial Growth						
Clinical Mastitis	66 (54, 78)	90 (85, 95)	19	64	10	7
CMT-Positive	37 (15, 59)	99 (98, 100)	2	94	3	1

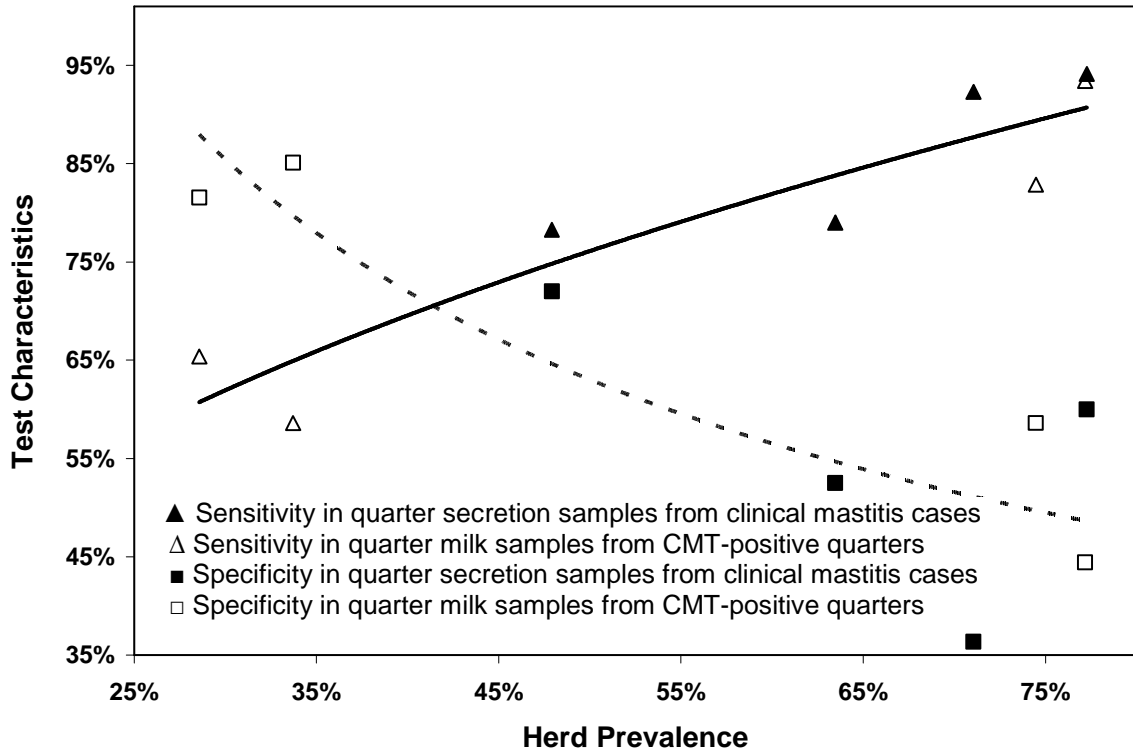
^a Percentage of samples identified correctly by the Bi-Plate Minnesota Easy Culture System. The initials TP represent the true positives and the initials TN represent the true negatives

^b Percentage of samples identified incorrectly by the Bi-Plate Minnesota Easy Culture System. The initials FP represent the false positives and the initials FN represent the false negatives

^c Quarter secretion samples from clinical mastitis cases

^d Quarter milk samples from CMT-positive quarters from cows after parturition

Figure 6. 1. Test characteristics of the Minnesota Easy Culture Bi-Plate System to identify bacterial growth in milk samples from 8 herds that differ in prevalence of bacterial growth.



Sensitivity and specificity of the Minnesota Easy Culture Bi-Plate System to identify bacterial growth are represented (▲) and (■), respectively, in quarter secretion samples from clinical mastitis cases in 4 herds, and represented (△) and (□), respectively, in quarter milk samples from CMT-positive quarters from cows after parturition in the other 4 herds.

Definition of Herd Prevalence:

For the 4 clinical mastitis study herds: The proportion of clinical quarter samples submitted that yielded a positive laboratory culture result.

For the 4 CMT-positive postparturient mastitis study herds: The proportion of CMT-positive quarters from post-parturient cows that yielded a positive laboratory culture result.

CHAPTER VII

GENERAL SUMMARY

The goal of this research project was to evaluate of the efficacy of on-farm programs for the diagnosis and selective treatment of clinical and subclinical mastitis in dairy cattle. This chapter will justify the need for this research; state the objectives pursued; summarize the methods, results and conclusions; state the implications or significance of these findings to the industry; and discuss future directions for research that still need pursuing.

Clinical and subclinical mastitis in dairy cattle is a common disease that has significant ramifications, including financial losses to dairy farmers, adverse effects on cow health and welfare and potential influences on public health. There is a need to develop and validate new management strategies to reduce its impact on cow health and dairy production economics, while continuing to ensure the quality and safety of dairy food products. The review presented in Chapter I discussing the epidemiology and treatment of clinical mastitis during lactation, and subclinical mastitis after parturition, brings to light opportunities in how treatment decisions are made on farms, and introduces potentially useful new on farm diagnostic tools. The next few paragraphs summarize that review.

It has been reported that more than half of cultures from clinical mastitis cases yield no bacterial growth or growth of Gram-negative bacteria. These cases of clinical mastitis may not benefit from IMM antibiotic therapy. Conversely, Gram-positive intramammary infections (IMI) benefit from intramammary therapy (IMM). The selective treatment of clinical mastitis based on culture results might reduce treatment related costs and promote more judicious use of antibiotics.

Subclinical mastitis at calving is highly prevalent, and has important consequences in cow health and production throughout the future lactation. The successful identification and treatment of those infections immediately after parturition, in the period before milk is saleable, has the potential to diminish the economic impact of disease, while treatment related costs are reduced. However, similar to clinical mastitis cases, only Gram-positive IMIs may benefit from antibiotic therapy.

Finally, in order to identify quarters affected with mastitis that may benefit from IMM antibiotic therapy and to make judicious use of antibiotics a reality, there is a need to develop and validate tools that will allow producers to make a rapid and accurate on-farm diagnosis of the presence and etiology of infection. Qualitative measures of somatic cell count such as the California Mastitis Test (CMT), though possible a useful screening tool, is unlikely to fit this purpose because of the a) high false positive rate to detect IMI in the immediate post-parturient period, and b) the CMT fails to classify the type of pathogen present. On farm culture (OFC), a rapid and inexpensive tool, could conceivably be used to a) diagnose and guide strategic treatment of clinical mastitis

cases, and b) use in conjunction as a screening tool (such as CMT), to diagnose and guide strategic treatment of subclinical IMI in cows after parturition.

Two multi-state multi-herd clinical trials were carried out. One trial evaluated the efficacy of the selective treatment of clinical mastitis during lactation based on on-farm culture results. A second trial evaluated the efficacy of the use of the CMT alone, or using the CMT and an OFC system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition.

CLINICAL TRIAL I: EFFICACY OF THE SELECTIVE TREATMENT OF CLINICAL MASTITIS DURING LACTATION BASED ON ON-FARM CULTURE RESULTS

The use of on-farm milk culture for the selective treatment of clinical mastitis may represent a tremendous opportunity to reduce antimicrobial use on commercial dairy farms without sacrificing the efficacy of treatment or the long-term health and production potential of the cow. Benefits could include reduced economic cost of therapy, reduced risk of antimicrobial residues in milk, and a reduction in the potential risk for development of antimicrobial resistance in mastitis pathogens. The problem is that studies have been lacking to describe efficacy and cost benefit of using on farm culture for the selective treatment of clinical mastitis.

The objective of this study was to investigate the efficacy of using an OFC system to guide strategic treatment decisions in cows with mild and moderate clinical mastitis. Outcomes evaluated included: a) risk to receive primary IMM antibiotic therapy because of study assignment, b) risk to receive secondary (or extended) IMM antibiotic therapy, c) days to return to visibly normal milk (days to clinical cure), d) days of milk withheld from market (days out of the tank), e) risk for bacteriological cure within 21 days of enrollment, f) new intramammary infection risk within 21 days of enrollment, g) presence of infection, clinical mastitis recurrence, or risk of removal from herd (ICR) within 21 days of enrollment; and over the remainder of the lactation (up to 365 days post-enrollment): h) risk and days to a recurrence of a clinical mastitis event in the same quarter, b) somatic cell count, i) milk production, and j) cow survival post-enrollment (culling and death events).

Four hundred and twenty two cows affected with mild or moderate clinical mastitis in 449 quarters were randomly assigned to either a) a positive-control treatment program or b) an on-farm culture-based treatment program. Quarter cases assigned to the positive-control group received immediate on-label IMM treatment with Cephapirin Sodium. Quarters assigned to the culture-based treatment program were not treated until the results of on-farm culture were determined after 24 hr of incubation. Quarters in the culture-based treatment program that showed Gram-positive growth or a mix infection were treated according to label instructions using IMM Cephapirin Sodium. Quarters assigned to the culture-based treatment program that showed Gram-negative or no growth did not receive IMM therapy.

Chapter II reports on short-term outcomes of this study. The proportion of quarters that received IMM antibiotic therapy because of study assignment was 100% and 44% in quarter cases assigned to the positive-control and culture-based treatment groups, respectively. The proportion of quarters that received secondary (or extended) antibiotic therapy was 36% and 19%, for cases assigned to the positive-control and to the culture-based treatment groups, respectively. There was a tendency for a reduction in days out of the tank for the milk from cows assigned to the culture-based treatment program *vs.* cows assigned to the positive-control group (5.9 *vs.* 5.2 days). There were no significant differences between cases assigned to the positive-control and cases assigned to the culture-based treatment program in days to clinical cure (2.7 *vs.* 3.2 days), bacteriological cure risk within 21 days of enrollment (71 *vs.* 60%), new intramammary infection risk within 21 days of enrollment (50 *vs.* 50%) and presence of infection, clinical mastitis recurrence, or risk of removal from the herd (ICR risk) (68 *vs.* 71%) within 21 days after the clinical mastitis case.

Chapter III reports on long-term outcomes of this study. There were no significant differences between cases assigned to the positive-control and cases assigned to the culture-based treatment program in the risk and days for recurrence of clinical mastitis in the same quarter (35% and 78 days *vs.* 43% and 82 days), linear somatic cell count (4.2 *vs.* 4.4), daily milk production (30.0 *vs.* 30.7 kg), and the risk and days for culling or death events (28% and 160 days *vs.* 32% and 137 days) for the rest of the lactation after enrollment of the clinical mastitis case.

The use of an OFC system to guide the strategic treatment of clinical mastitis reduced IMM antibiotic use by half and tended to reduce milk withholding time by one day. Discarded milk following clinical mastitis is one of the major economic losses of the clinical disease; any reduction in the time that milk is withheld from the market has an important impact on the disease economic consequences. These revenues and the additional expenses resulting from implementing the OFC program will be used to evaluate the overall cost-benefit of using an OFC system to guide strategic treatment decisions in cows with mild and moderate clinical mastitis. In order to add a risk dimension to the deterministic analysis and have an understanding of the important factors that make the culture-based program profitable, a sensitivity and breakeven analysis will be completed. In addition, a stochastic analysis will be conducted to account for the uncertainty associated with the probability distribution on inputs. This will evaluate the probability distribution of the two programs' performance and evaluate how the output (i.e. program response) varies depending on the variation of inputs.

The public health impact of reducing antibiotic use for the treatment of clinical mastitis, due to risk avoidance of antibiotic residue violations, could by far outweigh the on-farm cost savings derived from a reduction in antibiotic use. Resistance to antibiotics associated with the use of antibiotics in food animals is currently an issue of principal concern. If half of all antimicrobial drugs used in dairy farms are dedicated to clinical mastitis treatment, the selective treatment of clinical mastitis based in OFC results has the potential to reduce total antibiotic use on dairy farms by 25%.

One potential limitation of this study is that the label of IMM antibiotic administered in this study, Cefa-Lak[®] (Fort Dodge Animal Health, Fort Dodge, IA) does not include efficacy claims against Gram-negative bacteria. Two IMM antibiotic preparations, Hetacin-K[®] (Fort Dodge Animal Health, Fort Dodge, IA) and Spectramast[®] (Pfizer Animal Health, New York, NY), are currently approved in the USA with a label that claims efficacy against clinical mastitis in lactating dairy cattle associated with *Escherichia coli*. However, published peer reviewed studies are lacking that reporting the efficacy of these two antimicrobial formulations to treat clinical mastitis cases where *Escherichia coli* or other Gram-negative pathogens are isolated. There is a need for controlled field trials evaluating their efficacy in treating clinical mastitis. Furthermore, clinical mastitis selective treatment programs based in OFC culture results using these IMM antibiotic preparations should be evaluated. Until this scientific knowledge becomes available, the validity of the present study results when using antibiotics other than Cephapirin Sodium is not known.

In addition to the aforementioned objectives, there exists an opportunity to use data collected in this project to describe cow and quarter predictors of a clinical mastitis cure using the different outcomes measured. Examples of those predictors that could be incorporated into a history-based approach to clinical mastitis therapy decision making include cow parity, days in milk (DIM) at clinical mastitis event, previous occurrence of a clinical mastitis case in the same quarter in the present lactation, severity of the case, number of quarters affected, and current case etiology of infection as determined by

OFC. Decision making dynamic programming that consider these predictors could be used to determine the optimal case management decision for a cow with clinical mastitis (treat her, do not treat her, keep her or cull her) depending on her net present value and the probability distribution of the different outcomes evaluated.

On-farm culture may also have other potential applications to guide treatment decisions than selective treatment. There may be an opportunity to use OFC to decide the antibiotics to use and duration of therapy. Studies evaluating extended IMM antibiotic therapies found they may result in better bacteriological cure risk when treating clinical and subclinical infections caused by non-agalactiae streptococci and *Staphylococcus aureus* (Gillespie *et al.*, 2002, Oliver *et al.*, 2002; Oliver *et al.*, 2003). The use of a longer duration treatment of induced *Streptococcus uberis* infections has been shown to result in bacteriological cure rates that exceed 90% (Hillerton and Kliem, 2002).

Coagulase positive staphylococci colonies, of which *Staphylococcus aureus* is the most common mastitis pathogen, can be identified in the Factor agar half of the Bi-plate OFC because of a clear halo of β -hemolysis. In order to identify streptococci on-farm, the Tri-plate system, which is a plate that also includes a section of MTKT agar which is selective for streptococci, can be used.

Another potential application for OFC systems is the identification for treatment of other subclinically infected quarters when clinical mastitis is detected in one or more quarters. Simultaneous treatment of both clinically and subclinically infected quarters could result in a reduction in the prevalence of IMIs and would not result in increased milk discarded

in comparison to the treatment of the clinically affected quarter alone. After IMM antibiotic therapy milk is withheld from the market from all quarters and the withdrawal period is independent of the number of quarters treated. This hypothesis requires study.

Another novel application of OFC could be to monitor the bacteriological cure outcome of clinical mastitis cases treated on-farm. Although this application does not pursue a primary clinical mastitis treatment decision per se, OFC may be useful to guide the duration of antibiotic therapy or switching antibiotics depending if bacteria was eliminated or not, or if there was a significant reduction in the number of colony forming units. The efficacy and cost-benefit for this potential application of OFC requires further research.

CLINICAL TRIAL II: EFFICACY OF THE USE OF THE CMT ALONE, OR CMT AND AN OFC SYSTEM IN SERIES, TO DIAGNOSE AND GUIDE TREATMENT DECISIONS IN COWS WITH SUBCLINICAL MASTITIS AFTER PARTURITION

The use of the CMT and on farm culture for diagnosis and treatment of subclinical infections in cows after parturition before milk is saleable, has the potential to diminish the disease health and production impact throughout the future lactation. Benefits could include a reduction in clinical mastitis flare-ups, lower somatic cell count, reduction in milk losses associated with subclinical mastitis, improve reproductive performance and decrease risk of premature culling. However, many of these potential benefits need further study to confirm and quantify the nature of these proposed benefits.

The objective of this study was to investigate the efficacy of using the CMT alone, or CMT and an OFC system in series, to diagnose and guide treatment decisions in cows with subclinical mastitis after parturition. Outcomes evaluated included: a) quarter and cow risk to receive IMM antibiotic therapy because of study assignment, b) days of milk withheld from market (days out of the tank), c) bacteriological cure within 21 days of enrollment, d) new intramammary infection risk within 21 days of enrollment, and e) presence of infection, risk of clinical mastitis, or risk of removal from herd (ICR) within 21 days of enrollment, f) risk of clinical mastitis, g) somatic cell count, h) milk production, i) risk of conception, and j) risk to removal from herd during lactation.

A total of 1,885 cows from 14 herds were screened for enrollment into the study at 1-4 days after parturition. Of those, 1,168 cows which had a negative CMT result on all four quarters were not assigned to any treatment group. A total of 717 cows with at least one CMT-positive quarter were randomly assigned to either a) a negative-control group (NC), b) a CMT-based treatment group (CMTB), or c) to a culture-based treatment group given a CMT-positive result (CB|CMT-pos). Quarters from cows assigned to NC did not receive IMM antibiotic treatment. CMT-positive quarters from cows assigned to CMTB received immediate on-label IMM treatment with Cephapirin Sodium. Quarters from cows assigned to CB|CMT-positive were not treated until the results of on-farm culture were determined after 24 hr of incubation. Quarters from cows assigned to CB|CMT-positive showed Gram-positive growth were treated according to label instructions using

IMM Cephapirin Sodium. Quarters assigned to CB|CMT-positive showed no growth, Gram-negative or a mixed infection did not receive IMM therapy.

Chapter IV reports on short-term outcomes of this study. The use of the CMT to identify cows and quarters for the strategic treatment with IMM Cephapirin Sodium of subclinical IMI after parturition required the administration of IMM treatment to 49% of the quarters from cows with at least a CMT-positive quarter (38% of cows after parturition) and extended the time that milk is withheld from the market from 1.7 to 6.3 days. The selection for treatment of only CMT-positive quarters where Gram-positive bacteria was isolated using OFC required less antibiotic use (15% of the quarters and 40% of the cows with at least a CMT-positive quarter) and days out of the tank (4.4 days). The odds for a bacteriological cure within 21 days of enrollment were significantly higher for quarters of cows assigned to CMTB and tended to be higher for quarters of cows assigned to CB|CMT-positive. The proportion of quarters with bacteriological cure was 59, 50 and 42% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively. Using NC as the reference, there was no significant difference in the risk for new IMI for quarters from cows assigned to CMTB. The proportion of quarters with a new IMI was 44, 46 and 45% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the ICR risk (where ICR risk represented the presence of infection risk, clinical mastitis risk, or removal from herd risk within 21 days after enrollment) for quarters from cows assigned to CMTB and to CB|CMT-pos. The ICR risk was 53, 58 and 59% for quarters of cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Additionally, long-term outcomes of the intervention were evaluated in Chapter V since they represent the overall economic impact of the intervention. In this study, both treatment programs were effective on direct udder health outcomes, clinical mastitis and LSCC. The risk and average days after enrollment to a clinical mastitis event in the same quarter was 10% and 124 days, 8% and 114 days and 12% and 124 days for quarters assigned to CMTB, CB|CMT-pos and to NC, respectively. Similarly, the LSCC was numerically lower for cows assigned to CMTB than for cows assigned to NC. However, LSCC, although numerically lower, was not significantly lower for cows assigned to CB|CMT-positive. The average LSCC was 2.9, 3.1 and 3.3 for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. Using NC as the reference, there was no significant difference in milk production for cows assigned to CMTB and to CB|CMT-positive. The average lactation milk production was 35.1, 34.5 and 35.6 kg per day for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the pregnancy hazard risk ratio for cows assigned to CMTB or to CB|CMT-positive]. The risk and average days after enrollment to conception was 76% and 126 days, 75% and 119 days and 73% and 119 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively. There was no significant difference in the hazard risk ratio for removal from the herd for cows assigned to CMTB or to CB|CMT-positive. The risk and average days after parturition to removal from herd was 27% and 124 days, 24% and 144 days and 31% and 121 days for cows assigned to CMTB, CB|CMT-pos and to NC, respectively.

Increased incomes from the implementation of both programs, fewer clinical mastitis cases and lower LSCC, in addition to the additional expenses resulting from implementing both programs, extra labor cost, CMT and OFC implementation costs, antibiotic expenses and more days of milk withheld from the market will be used to evaluate the overall economic impact of using the CMT and an OFC system to guide strategic treatment decisions in cows after parturition. Deterministic and stochastic models will be used for a cost-benefit and risk (helpful for decision making) analysis. In addition to the economic implications, the intervention has direct repercussions in animal welfare by improving udder health and reducing animal suffering due to less clinical mastitis during lactation.

Future analysis from data collected in this clinical trial will add to the already existing literature validating the use of the CMT as a cow-side screening tool to detect bacterial infections in quarter milk samples in the first three days after parturition. In addition, it will be validated the use of the CMT as a cow-side screening tool followed by on-farm culture in CMT-positive quarters to a) detect bacterial infections in quarter milk samples in the first three days after parturition, and to b) diagnose Gram-positive bacterial infections in quarter milk samples in the first three days after parturition.

Multi-herd studies reporting prevalence and dynamics of infection in the immediate postparturient period are scarce. Therefore, there is the opportunity to use available data to describe quarter IMI status at parturition, as well as infection dynamics in early lactation. There is also the need to define the association between udder infection status

defined by milk bacteriological culture and etiology of infection in the immediate postparturient period and milk production, SCC, incidence of clinical mastitis, reproductive performance, and culling for the rest of the lactation for both heifers and mature cows. Quantifying the association between bacteriological cure or the development of an IMI in early lactation with milk production, milk quality, as well as udder health, reproductive, and cow survival parameters, will serve to provide an estimation of the economic impact of an intervention aimed at reducing the prevalence of IMI at parturition.

In addition to the immediate postpartum period, there may be other potential opportunities to use OFC alone, or in conjunction with a screening tool (such as CMT or DHIA SCC), to diagnose and guide strategic treatment of subclinical IMI. Examples may include the identification and treatment of subclinical IMI during lactation or selective dry cow therapy at time of dry off. However, the efficacy and cost-benefit of these interventions requires further research.

As for clinical mastitis, there is the opportunity to evaluate the use the Tri-plate OFC system to extend the IMM antibiotic therapy when treating subclinical infections caused by non-agalactiae streptococci and *Staphylococcus aureus* since it has been proven that higher bacteriological cures are achieved with longer duration therapies (Oliver *et al.*, 2002; Oliver *et al.*, 2003).

A secondary objective of both studies, reported in Chapter VI, was to validate the use of the Minnesota Easy Culture Bi-Plate System for two different applications; diagnose on farm IMIs in quarter milk samples from clinical mastitis cases; and diagnose on farm subclinical IMI in CMT-positive quarter milk samples collected after parturition.

Agreement beyond chance with laboratory culture results, test characteristics and predictive values were described using three diagnostic interpretations of the bi-plate culture results: a) identification of Gram-positive or Gram-negative bacterial growth (*vs.* no growth), b) identification of Gram-positive bacterial growth (*vs.* no growth and Gram-negative growth), and, c) identification of Gram-negative bacterial growth (*vs.* no growth and Gram-positive growth). A total of 193 quarter milk samples from clinical mastitis cases from the 8 herds and 430 CMT-positive quarter postparturient milk samples from the 14 herds were cultured both on farm using the on-farm culture system and at the laboratory using standard bacteriological culture procedures.

The agreement beyond chance, Kappa, between both culture methodologies to correctly classify samples as no-growth, Gram-positive, Gram-negative and mix infections was moderate, 51% in clinical mastitis and 44% in CMT-positive samples. Kappa for the three diagnostic classifications was 44, 61 and 57%, respectively, in clinical mastitis samples; and 45, 44 and 45%, respectively, in CMT-positive postparturient samples. The sensitivity of the bi-plate on-farm culture system for the three diagnostic classifications was 87, 78 and 73%, respectively, in clinical mastitis samples; and 64, 58 and 64%, respectively, in CMT-positive postparturient samples. The specificity of the bi-plate on-farm culture system for the three diagnostic classifications was 55, 83 and 87%,

respectively, in clinical mastitis samples; and 81, 85 and 97%, respectively, in CMT-positive postparturient samples. The predictive value of a positive result for the three diagnostic classifications was 79, 74 and 66%, respectively, in clinical mastitis samples; and 75, 74 and 37%, respectively, in CMT-positive postparturient samples. The predictive value of a negative result for the three diagnostic classifications was 69, 86 and 90%, respectively, in clinical mastitis samples; and 72, 74 and 99%, respectively, in CMT-positive postparturient samples.

We concluded that the Minnesota Easy Culture Bi-Plate System is a useful cow-side test to correctly identify bacterial growth, Gram-positive bacterial growth, or Gram-negative bacterial growth in quarter secretion samples from clinical mastitis cases and in CMT-positive quarter milk samples collected after parturition. Treatment decisions based on identification of bacterial growth, or GP bacterial growth specifically, were correct over 73% of the time. Whether or not this level of accuracy is sufficient will be determined by considering the short- and long-term outcomes for the cow, the impact on antibiotic use, and overall cost-benefit of the two programs evaluated.

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