2009

Mathematical and Molecular Epidemiology of Subclinical Mastitis Treatment in Lactating Dairy Cows

John Barlow

University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Recommended Citation

Barlow, John, "Mathematical and Molecular Epidemiology of Subclinical Mastitis Treatment in Lactating Dairy Cows" (2009). 
Graduate College Dissertations and Theses. 16. 
https://scholarworks.uvm.edu/graddis/16

This Dissertation is brought to you for free and open access by the Dissertations and Theses at ScholarWorks @ UVM. It has been accepted for inclusion in Graduate College Dissertations and Theses by an authorized administrator of ScholarWorks @ UVM. For more information, please contact donna.omalley@uvm.edu.
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy specializing in Animal, Nutrition and Food Sciences

Dissertation Examination Committee:

Advisor
Ynte H. Schukken, D.V.M., Ph.D.

A. John Bramley, Ph.D.

Takamaru Ashikaga, Ph.D.

Thomas B. McFadden, Ph.D.

Ruth N. Zadoks, D.V.M., Ph.D.

Chairperson
Burton William Wilcke Jr., Ph. D.

Vice President for Research and Dean of Graduate Studies
Frances E. Carr, Ph. D

Date: September 12, 2008
Abstract

Subclinical mastitis remains the dominant form of mastitis affecting dairy cattle, and is responsible for the greatest economic losses associated with mastitis in dairy herds in the major dairy producing countries. Mastitis control has relied on a series of well established management practices that have led to significant improvements in mastitis prevalence and milk quality measures over the past 3 decades. Changes in pathogen prevalence, including the shift in absolute and relative importance of pathogens such as *Escherichia coli*, *Klebsiella* spp. and *Streptococcus uberis* combined with the ongoing importance of *Staphylococcus aureus*, highlight the need for continued research to evaluate mastitis control practices.

This dissertation examines targeted antimicrobial treatment of chronic subclinical mastitis during lactation as a control measure implemented in herds that have applied standard mastitis control practices. Treatment of subclinical mastitis caused by the major gram-positive mastitis pathogens *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* in dairy herds is examined. Methods include predictive modeling of the effects of lactation therapy using a deterministic state-transition model of pathogen transmission dynamics, and evaluation of lactation treatment in a negative controlled clinical field trial. Concepts put forward include the distinction between direct and indirect effects of mastitis control practices and the value of molecular diagnostics to improve our understanding of mastitis epidemiology and the impact of control programs.

Results obtained from predictive modeling indicate that overall positive population level effects of lactation therapy would be realized for herds that have successfully implemented practices that reduce pathogen transmission. A novel finding was the prediction that under management scenarios with high pathogen transmission rates treatment of subclinical mastitis will have little impact on the proportion of infected quarters and no positive population level effect in reducing new infection rates. In a field trial, positive direct and indirect effects of treatment of *S. aureus* mastitis were observed suggesting benefits of lactation therapy targeting this pathogen. Potential benefits were off-set by the frequent finding of post treatment infections which resulted in no improvement in somatic cell count of treated cows compared to untreated controls. Lactation therapy of *S. uberis* and *S. dysgalactiae* mastitis resulted in cure proportions and duration of infection did not differ from spontaneous cure of untreated controls and there was limited evidence of an effect of treatment on rates of new infection. A unique finding was the identification of an association between *Coxiella burnetii* shedding and subclinical mastitis in dairy cattle.

It is concluded from this research that treatment of subclinical mastitis during lactation may be justified under specific conditions, and it is recommended that dairy farm managers and their advisors should not routinely implement lactation therapy of subclinical mastitis without careful consideration of the potential benefits and risks.
Citations

Material from this dissertation has been published in the following form:


Material from this dissertation has been accepted for publication in Preventive Veterinary Medicine on September 26, 2008 in the following form:

Table of Contents

Citations .......................................................................................................................... ii

List of Tables .................................................................................................................... ix

List of Figures ................................................................................................................... xi

List of Abbreviations ........................................................................................................ xii

Chapter 1 – Introduction

Literature Review .............................................................................................................. 1

Subclinical mastitis: Identification, causes and significance for dairy herds ............. 1

Mastitis control on dairy farms: current practices ......................................................... 6

Mastitis treatment in dairy cattle and treatment of subclinical mastitis during lactation .................................................................................................................. 8

Factors associated with the decision to implement subclinical mastitis therapy during lactation ................................................................................................................. 10

Further rationale for subclinical mastitis control: Indirect effects of therapy ......... 15

Evaluating interventions at the population level: Use of deterministic models ...... 16

Evaluating interventions at the molecular level: Use of molecular epidemiology in field trials .................................................................................................................. 18

Molecular methods of bacterial strain typing ................................................................. 24

Outline of this Dissertation .............................................................................................. 26
Chapter 2 – A mathematical model demonstrating indirect and overall effects of lactation therapy targeting subclinical mastitis in dairy herds

Abstract ........................................................................................................................................29

Introduction ...................................................................................................................................30

Methods..........................................................................................................................................32

  Model development ..................................................................................................................32

  Entry into the lactating herd .................................................................................................37

  Exits from the lactating herd .................................................................................................37

  New infection rates ..................................................................................................................39

  Spontaneous cure rate of subclinical intramammary infections ......................................42

  Cure rates associated with the treatment of chronic subclinical mastitis ......................43

  Estimating the effect of treatment of chronic subclinical mastitis during lactation ........45

  Model behavior and scenario analysis ...............................................................................46

    Simulations, sensitivity and uncertainty analysis associated with key parameters ....47

Results ...........................................................................................................................................48

Discussion ....................................................................................................................................56

Conclusion ....................................................................................................................................60

Acknowledgements ..................................................................................................................61

References .....................................................................................................................................62
Chapter 3 - Effects of Lactation Therapy on *Staphylococcus aureus* Mastitis
demonstrated through a Field Trial and Molecular Strain Typing

Abstract ..........................................................................................................................67

Introduction ..................................................................................................................69

Materials and Methods...............................................................................................72

Study design..................................................................................................................72

Milk sample collection and bacteriologic analysis .......................................................74

Infection status..............................................................................................................75

Treatment program......................................................................................................77

Species identification and *S. aureus* strain typing.......................................................79

Duration of infection....................................................................................................82

Statistical methods......................................................................................................83

Results...........................................................................................................................86

Intramammary infections eligible for therapy in treated and control groups ..........86

Direct effect of treatment on intramammary infection prevalence.............................90

Direct effect of treatment on duration of *S. aureus* intramammary infection.........95

Effect of treatment on *S. aureus* clinical mastitis rates and culling due to mastitis 97

Indirect effects of treatment on *S. aureus* intramammary infection incidence ......98

Strain typing ................................................................................................................98

Influence of strain typing on determination of bacteriologic cure ..............................99

New intramammary infections in recovered susceptible quarters ............................100

Effect of extended therapy treatment on somatic cell count ....................................101
S. aureus transmission parameter estimates .......................................................... 104

Discussion .................................................................................................................. 105

Conclusion .................................................................................................................. 115

Acknowledgements .................................................................................................... 115

References .................................................................................................................. 116

Chapter 4 - Effects of lactation therapy on subclinical Streptococcus uberis and
Streptococcus dysgalactiae mastitis

Abstract ...................................................................................................................... 121

Introduction ............................................................................................................... 123

Materials and Methods ............................................................................................ 129

Study design .............................................................................................................. 129

Milk sample collection and bacteriologic analysis ................................................... 131

Treatment program .................................................................................................. 133

Infection status ......................................................................................................... 135

Duration of infection ................................................................................................. 139

Species identification and S. uberis strain typing ..................................................... 140

Antimicrobial susceptibility testing .......................................................................... 142

Statistical methods ................................................................................................... 144

Results ....................................................................................................................... 145

Intramammary infection cure proportions in treated and control groups ............. 146

Effect of treatment on duration of S. uberis and S. dysgalactiae mastitis ............. 154
Effect of treatment on intramammary infection prevalence and incidence ..........156

New intramammary infection in recovered susceptible quarters following extended therapy .................................................................................................................................159

Diversity of S. uberis strains .................................................................................................................................160

Influence of speciation and strain typing on determination of IMI definition and bacteriologic cure ...............................................................................................................................................160

Association between antimicrobial susceptibility and bacteriologic cure........161

Discussion .................................................................................................................................................................165

Conclusion.................................................................................................................................................................169

Acknowledgements..................................................................................................................................................170

References.................................................................................................................................................................170

Chapter 5 - Association between Coxiella burnetii shedding in milk and subclinical mastitis in dairy cattle

Abstract .................................................................................................................................................................177

Introduction .............................................................................................................................................................178

Materials and methods ........................................................................................................................................181

Herd description ..................................................................................................................................................181

Coxiella burnetii PCR ...........................................................................................................................................181

Milk sampling, aerobic mastitis microbiology, and somatic cell count analysis ...182

Definitions ..............................................................................................................................................................183

Study design and statistical analysis ................................................................................................................184
List of Tables

Table 2. 1 Definitions of variables and initial parameter estimates for the subclinical mastitis model in a population of udder quarters .................................................................35

Table 2. 2 Estimates of the proportion of new intramammary infections caused by major gram positive pathogens that survive > 30 days obtained from literature review. ............42

Table 2. 3 Model realization of outcome variables for fixed parameter estimates. ....48

Table 2. 4 Indirect and overall effect estimate values observed for model realizations...55

Table 3. 1 Herd and study pen group descriptive data. ..................................................87

Table 3. 2 Staphylococcus aureus intramammary infections eligible for extended lactation therapy ........................................................................................................88

Table 3. 3 Staphylococcus aureus intramammary infections not eligible for extended lactation therapy ........................................................................................................89

Table 3. 4 Strain specific dynamics of Staphylococcus aureus intramammary infections. ................................................................................................................................94

Table 3. 5 Regression coefficients for the effect of treatment on intramammary infection prevalence and new intramammary infection incidence. ........................................95

Table 3. 6 Regression coefficients for the effect of treatment group on 45 day post treatment SCC. .................................................................................................................103

Table 3. 7 Transmission coefficient ($\beta$) estimates for Staphylococcus aureus transmission on 2 dairy farms......................................................................................................104
Table 4. 1 Frequency of *Streptococcus uberis* intramammary infections in treatment and control groups on Farm 1.................................................................150

Table 4. 2 Frequency of *Streptococcus dysgalactiae* intramammary infections in treatment and control groups on Farm 1.................................................................151

Table 4. 3 Frequency of *Streptococcus uberis* intramammary infections in treatment and control groups on Farm 2.................................................................152

Table 4. 4 Frequency of *Streptococcus dysgalactiae* intramammary infections in treatment and control groups on Farm 2.................................................................153

Table 4. 5 Duration of infection estimates for *Streptococcus uberis* and *Streptococcus dysgalactiae* intramammary infections........................................155

Table 4. 6 Antimicrobial susceptibility profile of *Streptococcus* spp. isolates obtained pre-treatment. .........................................................................................................................164

Table 5. 1 Frequency of *Coxiella burnetii* positive and negative cows ..........................189

Table 5. 2 Parameter estimates for the multivariate model of the association between *Coxiella burnetii* PCR status and the 3 month average linear somatic cell count score .190

Table 6. 1 Frequency of *Staphylococcus aureus* strain types isolated from 2 dairy herds including pulsed-field gel electrophoresis (PFGE) type and multilocus strain type (MLST) groupings.................................................................204
List of Figures

Figure 1.1 The pattern of intramammary infection status..............................................4
Figure 1.2 The balance of the decision to treat subclinical mastitis during lactation ....11
Figure 1.3 Time line of mastitis dynamics..................................................................12

Figure 2.1 Flow diagram of the state-transition model of subclinical mastitis........33
Figure 2.2 Frequency distribution of transmission parameter ($\beta_S$).......................41
Figure 2.3 Scenario analysis......................................................................................49
Figure 2.4 Sensitivity analysis....................................................................................51
Figure 2.5 Impact of changes in transmission parameter ($\beta_S$) on new intramammary infections..............................................................52
Figure 2.6 Impact of changes in transmission parameter ($\beta_S$) on effect estimates.......54

Figure 3.1 Study design and sample collection procedures for treatment trial.............78
Figure 3.2 Prevalence and incidence of \textit{Staphylococcus aureus} intramammary infections. .................................................................................................................................91
Figure 3.3 Least squares mean estimates of prevalence of \textit{Staphylococcus aureus} intramammary infections. .................................................................................................................................92
Figure 3.4 Kaplan-Meier survival function of chronic \textit{Staphylococcus aureus} intramammary infections. .................................................................................................................................96
Figure 3. 5 Effect of treatment on somatic cell count ..................................................103

Figure 4. 1 Study design and sample collection procedures for treatment trial..............135
Figure 4. 2 Frequency distribution of *Streptococcus dysgalactiae* and *Streptococcus uberis* intramammary infections by duration of infection categories.................................154
Figure 4. 3 Kaplan-Meier survival function of *Streptococcus dysgalactiae* and *Streptococcus uberis* intramammary infections. .................................................................156
Figure 4. 4 Incidence and Prevalence of *Streptococcus uberis* intramammary infections stratified by farm and treatment group. .................................................................157
Figure 4. 5 Incidence and Prevalence of *Streptococcus dysgalactiae* intramammary infections stratified by farm and treatment group. ......................................................158
Figure 4. 6 Scatter plot for pirlimycin antimicrobial susceptibilities.............................163

Figure 5. 1 Least squares mean 3 month average linear somatic cell count score for *Coxiella burnetii* postive and negative cows stratified by aerobic intramammary infection status . ........................................................................................................191
List of Abbreviations

BTSCC = bulk tank milk somatic cell count
Hlb = beta hemolysin
IMI = intramammary infection
MLST = multilocus sequence typing
PFGE = pulsed-field gel electrophoresis
PMTD = post milking teat disinfectant
RAPD = random amplified polymorphic DNA
SCC = somatic cell count
Introduction

Literature Review

*Subclinical mastitis: Identification, causes and significance for dairy herds*

Mastitis continues to be recognized as the most common and costly disease affecting dairy cattle (DeGraves and Fetrow, 1993; Fetrow et al., 2000). Mastitis is an inflammation of the mammary gland, and while it may have a variety of causes, bacterial infections are the predominant cause of mastitis among dairy cattle (Philpot and Pankey, 1975; Watts, 1988; Wellenberg et al., 2002). To be clear, the term mastitis is a non-specific descriptor of inflammatory changes of the mammary gland for which there are numerous etiologies. From a practical perspective, mastitis of dairy cattle is the response of the gland to an infection typically acquired by invasion of bacterial pathogens through the teat orifice and teat canal (streak canal). Infectious organisms may also enter the gland by hematogenous or percutaneous routes (Kennedy and Miller, 1993), although these routes are considered significantly less important in the context of practical mastitis control. The stages of the infection process, (from contamination of the teat end with a potential pathogen, to penetration of the teat duct by the pathogen, and establishment of the pathogen in the gland), have been described with references to the value of specific mastitis control practices (Bramley and Dodd, 1984).
The term intramammary infection (IMI) is not strictly synonymous with the term mastitis, as IMI is more commonly used in the context of a defined etiology following completion of diagnostic culture procedures. IMI refers to infection of ductal and secretory glandular tissues (mammary gland parenchyma) and/or lumenal spaces (i.e. alveolar and ductal lumen, gland cistern, teat cistern). In perhaps the most comprehensive review of bacterial organisms associated with bovine mastitis, Watts (1988) listed 137 species. In comparison Philpot and Pankey (1975) reported on a broader range of 86 species and groups of microorganisms associated with bovine mastitis that included eukaryotes (e.g. yeast, fungi, and algae), many of the bacterial species described by Watts, and viruses. Wellenberg et al. (2002) have reviewed the viruses that either have been associated with mammary inflammation or isolated from mammary secretions. Similar to some of the viruses, a number of the bacterial species that have been isolated from bovine milk are associated with minimal inflammatory changes (e.g. *Mycobacterium* spp., including *M. bovis, M. tuberculosis*, and *M. paratuberculosis*, *Brucella* spp., *Salmonella* spp., and *Listeria* spp.) and are frequently found in cases of concurrent systemic infection caused by these organisms (Watts, 1988; Kennedy and Miller, 1993). In some cases the mammary gland may act as source of exposure for transfer of organisms to neonates or other potential hosts, as also occurs for some of the helminth parasites (Watts, 1988; Kennedy and Miller, 1993). Philpot and Pankey (1975) have suggested some organisms included in their review “are not true pathogens of the bovine udder,” but may be present as secondary invaders. These authors advise that
individual case reports describing isolation of organisms from mammary secretions often do not provide conclusive evidence of an association with mastitis.

The most common bacteria associated with mastitis include species of the Staphylococcaceae, Streptococcaceae, Enterococcaceae, Enterobacteriaceae and Corynebacteriaceae families. The organisms commonly isolated from cases of mastitis have been categorized as ‘major’ or ‘minor’ pathogens based on the extent of clinical symptoms, effect on inflammatory response, milk yield and milk composition (Griffin et al., 1977). Minor pathogens include the coagulase-negative staphylococci and the corynebacteria. Major pathogens include *Staphylococcus aureus*, the streptococci, and various gram-negative coliform bacteria (Griffin et al., 1977). Two of the most important species of bacteria that cause mastitis in dairy cattle are the gram-positive pathogens *Staphylococcus aureus* and *Streptococcus uberis* (Bramley and Dodd, 1984; Hillerton et al., 1995; Hillerton and Berry, 2005). Infections of the mammary gland, including those caused by *S. aureus* or *S. uberis*, may be symptomatic (clinical) or asymptomatic (subclinical). Further, individual infections can be of short (transient) or extended (persistent or chronic) duration. Frequently the definition of short versus long duration IMI is one of convenience driven by the sampling frequency of surveillance studies, although it appears to be commonly accepted that IMI of $< 1$ month (i.e. 28 to 30 days) may be regarded as a short duration, and an IMI of $\geq 1$ month would be defined as chronic (Schukken et al., 2003; St Rose et al., 2003). Finally, clinical cases may be characterized by the degree of severity, ranging from mild to severe based on both local and/or systemic signs (Wenz et al., 2006). Thus IMI may range in character from short
duration mild to severe clinical cases, or from short to long duration subclinical cases, and the categories of clinical and subclinical are not exclusive (Dodd and Neave, 1970) (figure 1.1). Persistent intramammary infections can be characterized by extended periods of subclinical mastitis punctuated by one or more clinical episodes (Dodd and Neave, 1970; Zadoks et al., 2002a). Chronic subclinical infections are known to persist for as long as the duration of a lactation and may persist across multiple lactations (Hillerton and Berry, 2003).

Figure 1.1  The pattern of intramammary infection status. Uninfected susceptible quarters become infected and infection is first observed as either clinical or subclinical (A). Infected quarters may transition into and out of the clinical and subclinical states (B), with subclinical mastitis being the dominant form. Spontaneous recovery is possible from either infected state to an uninfected susceptible state (C). Antimicrobial treatment of a clinical quarter may result in either clinical cure (D1) shifting the infection to a subclinical state, or bacteriologic cure (D2) moving the quarter to an uninfected state. The successful treatment of subclinically infected quarters shifts the quarter to an uninfected state (E). Adapted from Dodd and Neave (1970) and Zadoks et al. (2002b).
In most herds, subclinical mastitis is the dominant form, and is responsible for the greatest economic losses due to reduced milk production (Fetrow et al., 2000). Chronic subclinical infections have long been recognized as a major barrier in the control of mastitis on dairy farms (Bramley, 1984; Hillerton and Berry, 2005). Although subclinical mastitis is the dominant form affecting cows, it frequently goes undetected or untreated for extended periods by most dairy producers (Bramley and Dodd, 1984; Hillerton and Berry, 2003; Oliver et al., 2004). Subclinical mastitis can be detected by increases in milk somatic cell count (SCC). Milk somatic cells include lymphocytes, macrophages, polymorphonuclear leukocytes (neutrophils), and epithelial cells. Changes in the relative and absolute leukocyte (white blood cell) counts of milk can be used to distinguish infected and uninfected mammary glands or quarters (Leitner et al., 2000; Detilleux, 2004). Increases in milk SCC are associated with reduced milk production, and SCC has been used as a measure of herd or regional milk quality and mastitis prevalence. The recent USDA-APHIS National Animal Health Monitoring System (NAHMS) Dairy 2007 report, indicates that only 6.7% of U.S. dairy producers surveyed achieve a bulk tank milk SCC (BTSCC) of < 200,000 cells/ml. BTSCC was between 200,000 and 400,000 cells/ml for 37.1% of producers, with 83% of all milk shipped being less than 400,000 cells/ml (United States Department of Agriculture, 2008). Ott and Novak (2001) reported that herds with bulk tank milk SCC <200,000 cells/ml generated between $100 and $300 per cow per year more then herds in higher BTSCC categories, consistent with previous estimates of mastitis costs (DeGraves and Fetrow, 1993). The high prevalence of subclinical mastitis among dairy cattle is a critical health issue
resulting in significant losses in productivity. Many U.S. dairy producers have an opportunity to enhance milk quality and profitability by minimizing subclinical mastitis. This will become even more important when the U.S. decides to implement milk quality limits (e.g. BTSCC < 400,000 cells/ml) that are in line with most developed countries. The introduction of cow level SCC testing at regular intervals provides information that can be used by producers or their advisors to identify subclinically infected cows (Bramley and Dodd, 1984). A threshold of 200,000 cells/ml has been recommended to distinguish between uninfected and infected quarters or cows (Dohoo, 1991; Smith et al., 2001; Schukken et al., 2003; Hillerton and Berry, 2005) and results of sequential monthly SCC testing can be used to identify cows with chronic subclinical mastitis (Schukken et al., 2003).

Mastitis control on dairy farms: current practices

Current mastitis control recommendations are designed to reduce the duration of infections and prevent new infections (Neave et al., 1969; Dodd et al., 1977). A series of large field trials conducted by the National Institute for Research in Dairying (NIRD) and the Central Veterinary Laboratory of the UK (Dodd and Neave, 1970; Kingwill et al., 1970) demonstrated the efficacy of the “Five Point Mastitis Control Plan” in reducing incidence and duration of the most common mastitis pathogens of that period. Specific components of this program include: 1) appropriate treatment and record keeping of clinical mastitis cases, 2) application of post-milking teat disinfectants (PMTD), 3) use of long acting intramammary antibiotic preparations in all cows at the end of lactation (i.e.
“dry-cow therapy” at “dry-off”), 4) culling or segregation of ‘chronically’ infected cows, and 5) annual milking machine evaluation and repair. Bramley and Dodd (1984) have reviewed how specific components of this plan influence the risk for infection. The widespread adoption of the five-point mastitis control plan is likely in great part because farmers are able to apply the practices with limited increases in labor and no need to rely on diagnostic procedures (Dodd et al., 1977). The adoption of these control practices has led to the successful control of contagious pathogens, especially *Streptococcus agalactiae*, and the significant reduction in *Staphylococcus aureus* and *Streptococcus dysgalactiae*. This has resulted in reduced incidence of clinical mastitis and overall reductions in BTSCC on a national scale in the major dairy producing countries in both the Northern and Southern hemispheres over the past decades (Myllys et al., 1998; Bradley and Green, 2001; Forshell and Østerås, 2001; McDougall, 2002; van Schaik et al., 2002; Pitkälä et al., 2004; Hillerton and Berry, 2005; Piepers et al., 2007).

Concurrent with these changes, a number of authors have recognized a shift in the dominant species of bacteria causing clinical and subclinical mastitis over the past 40 years (Myllys et al., 1994; Bradley and Green, 2001; Forshell and Østerås, 2001; Bradley, 2002; Pitkälä et al., 2004; Hillerton and Berry, 2005; Sampimon et al., 2005; Piepers et al., 2007). Two important trends are worth note. First, while the prevalence of *S. aureus* has declined, this pathogen remains an important cause of mastitis, in many surveys representing the most frequently isolated major pathogen (Wilson et al., 1997; Makovec and Ruegg, 2003; Pitkälä et al., 2004; Sampimon et al., 2005; Østerås et al., 2006; Piepers et al., 2007). Second, the streptococcal species, especially *S. uberis*, and the
coli form bacteria, especially *Escherichia coli* and *Klebsiella* spp., have increased in relative importance as a cause of both clinical and subclinical mastitis (Bramley, 1984; Bradley and Green, 2001; Bradley, 2002; McDougall, 2002; Makovec and Ruegg, 2003; Hillerton and Berry, 2005; Dogan et al., 2006). Clearly there is continued opportunity to improve mastitis control, warranting investigations of practices that may be readily integrated with established control programs. The detection and treatment of subclinical mastitis cases during lactation has received some attention in this regard, although has been assumed not to be cost effective (McDermott et al., 1983; Hillerton and Berry, 2003). Recent work has suggested that treatment of subclinical mastitis cases during lactation may be economically beneficial under some management scenarios (Swinkels et al., 2005a; Swinkels et al., 2005b; Salat et al., 2008).

*Mastitis treatment in dairy cattle and treatment of subclinical mastitis during lactation*

Treatment of mastitis accounts for a major use of antimicrobials in dairy cattle and many current protocols for clinical mastitis may be ineffective (Hillerton and Kliem, 2002; Zwald et al., 2004; Sawant et al., 2005; Pol and Ruegg, 2007). Clinical mastitis is readily observed, and is frequently treated with the goal of returning milk to a normal marketable consistency (clinical cure) but often treatment is given without specific information on the cause of infection (Bramley and Dodd, 1984). Appropriate antimicrobial selection based on pharmacokinetic and pharmacodynamic principles must be considered when selecting drug, dose concentration, and dosing frequency to achieve minimum inhibitory concentrations at the site of infection. Commercially available
intramammary antimicrobial formulations are administered as an infusion through the teat canal using single dose syringes with specially designed applicator tips. Appropriately selected systemic therapies may be as efficacious as intramammary preparations (Sérieys et al., 2005; Salat et al., 2008; Sandgren et al., 2008), and lipid solubility appears to be a key factor affecting distribution to the mammary gland for drugs given systemically (Baggot, 2006). In the United States, only intramammary antimicrobial infusion formulations are currently approved for treatment of either clinical or subclinical mastitis.

For *S. uberis*, bacteriologic cure rates following treatment of clinical mastitis appear to be higher compared to treatment of subclinical mastitis (Zadoks, 2007), while for *S. aureus* subclinical treatments have shown similar efficacy compared to treatment of clinical cases (Barkema et al. 2006). Comparison of different studies evaluating treatment of clinical and subclinical IMI should be made with caution due to differences in enrollment criteria, infection definitions, and cure definitions.

Positive results associated with early treatment of cows infected with the major mastitis pathogens *S. aureus* and *S. uberis* (Sol et al., 1997; Sol et al., 2000; Hillerton and Kliem, 2002), have led to recommendations for intensive antimicrobial treatment protocols for subclinical mastitis in dairy herds. A number of studies have demonstrated the efficacy of treating subclinical mastitis caused by *S. aureus* or streptococcal species during lactation (Sol et al., 1997; Gillespie et al., 2002; Oliver et al., 2003; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005). Increased proportions of quarters with chronic subclinical mastitis are cured following extended therapy of 5 to 8 days
compared to either spontaneous cure of untreated quarters, or cure following label recommended 2-day duration of therapy. In many cases, cure rates for extended therapy exceeded 90% and were significantly improved over either the no-treatment rates of spontaneous cure (< 30%) or the two-day treatment cure rates (67-77%). These studies provide evidence of the potential efficacy of extended therapy for treatment of chronic subclinical mastitis caused by *S. aureus* or *Streptococcus* spp., however the decision to implement such a regimen needs careful consideration of the potential benefits and costs (Hillerton and Berry, 2003).

*Factors associated with the decision to implement subclinical mastitis therapy during lactation*

The circumstances that impact a decision to treat subclinical mastitis are similar to those that have been described for clinical mastitis (figure 1.2). However, the decision to treat subclinical mastitis is perceived as being less urgent and may be considered optional or discretionary. Historically, treatment of subclinical mastitis has been delayed until dry-off. The high efficacy of dry cow therapy for bacteriologic cure, and the reduced cost of dry cow therapy compared to the costs of discarded milk for lactation therapy, have influenced subclinical mastitis treatment decisions. Yet, as has been pointed out in a recent review, “the balance has changed where milk quality is often a significant component of price, and it may be that in some circumstances treating subclinical infections has a value, but these circumstances should be properly evaluated first” (Hillerton and Berry, 2003).
Direct benefits (cow effects)
- Increased cure rate and decreased duration of infection
- Reduced clinical mastitis cases
- Reduced individual SCC
- Increased welfare

Indirect benefits (herd effects)
- Reduced transmission and new infection rates
- Reduced herd SCC and improved milk quality

Costs of no treatment
- Delayed return to higher quality milk
- Increased risk of exposure to herd resulting in new or recurrent IMI
- Increased culling
- Decreased welfare

Costs of treatment
- Increased treatment costs (includes labor and diagnostics)
- Increased discarded milk
- Unnecessary discarded milk if treatment not needed for cure

Data needed to understand the balance (Swinkels et al. 2005)
- Probability of cure following therapy
- Probability of spontaneous cure, chronic IMI, and clinical mastitis
- Probability of transmission to other cows
- Potential for increased revenue (e.g. increased milk production, or increased milk quality premium payments)
- Potential for reduced costs (e.g. prevention of repeated or recurrent clinical mastitis, prevention of new IMI, reduced culling, reduced production losses)
- Reduced income (e.g. increased discarded milk)
- Additional costs, including labor, diagnostic and treatment costs

Figure 1.2 The balance of the decision to treat subclinical mastitis during lactation
Early detection and cure of subclinical mastitis cases may be beneficial in the individual as cure may reduce duration of infection and prevent subsequent clinical episodes, as well as be associated with a reduction in SCC, although there is currently limited evidence supporting this hypothesis. Two recent studies found a significant reduction in SCC following treatment of subclinical mastitis (St Rose et al., 2003; Sandgren et al., 2008), but only one of these two studies found a reduction in clinical mastitis rates among treated cows compared to an untreated control group (St Rose et al., 2003). The prevention of recurrent clinical episodes and associated clinical treatments may offset the additional antimicrobial use associated with treating subclinical cases (Figure 1.3).

Figure 1.3 Time line of Mastitis dynamics and potential influence of early detection and treatment of chronic cases. A mastitis case begins when a susceptible cow or quarter becomes infected. An individual infection persists for a variable duration of time dependent on pathogen, cow, and treatment factors. Each infection may include either a subclinical stage or a clinical stage or both clinical and subclinical periods. Treatment of subclinical mastitis (for example at 1) may have the greatest value if the overall effect of treatment includes a reduction in duration of infection and an associated reduction in somatic cell count, prevention of subsequent clinical episodes, and reduction of exposure of other susceptible individuals in a herd. Figure adapted from Halloran (1998).
An important concern in treatment of clinical and subclinical mastitis is selection of individual cases for therapy to avoid inappropriate or unwarranted use of antibiotics. This may be especially true for *Escherichia coli* infections given their transient nature (high spontaneous cure rate) (Hogan and Smith, 2003) and where there are no approved intramammary formulations indicated for treatment of subclinical gram-negative pathogens. Similarly, the short duration of many infections caused by gram-positive pathogens (Grommers et al., 1985; Todhunter et al., 1995; Watt, 1999; Zadoks et al., 2003), suggests that treatment should be delayed for a newly acquired subclinical infection in the absence of evidence of persistence. This, in combination with knowledge about the impact of chronic subclinical mastitis on milk production and SCC levels, suggests that chronic subclinical mastitis cases caused by major gram-positive pathogens (e.g. *S. aureus* and *Streptococcus* spp.) are leading candidates for lactation therapy. This also suggests that an accurate diagnosis is required for the appropriate and prudent use of lactation therapy.

There are few field studies examining the potential cost effectiveness of treating subclinical mastitis in dairy herds. Diagnosis and treatment of cows with *Streptococcus agalactiae* infections offers the clearest example of circumstances where the benefits of lactation therapy for subclinical mastitis have been described at the population level (Yamagata et al., 1987; Erskine and Eberhart, 1990). Successful treatment of subclinical *S. agalactiae* infections can be achieved using commercially available intramammary formulations at labeled dosing intervals, [e.g. 2 infusions of penicillin/novobiocin administered 24 hours apart (Erskine and Eberhart, 1990)]. The benefits of these ‘blitz’
treatment programs are attributed to high cure rates and prevention of contagious mastitis transmission, resulting in herd level reductions in SCC and increases in milk production (Erskine and Eberhart, 1990). The value of blitz therapy for control of other major pathogens has been described as being limited based on circumstances in field studies where the objective was to reduce herd level SCC from >700,000 cells/ml to below 400,000 cells/ml (Kingwill et al., 1970). In these studies the long-term reductions in mastitis prevalence were attributed to the effect of post milking teat disinfection and blanket dry-cow therapy consistently applied over a 12 to 24-month period. Further, in most scenarios any benefit of ‘blitz’ treatment is likely to be short-lived in the absence of consistent PMTD and blanket dry-cow therapy.

McDermott et al. (McDermott et al., 1983) provide results that argue against treatment of subclinical mastitis during lactation using SCC as the trigger for treatment without regard to bacteriologic diagnostics. These authors selected cows for treatment based on a single SCC measure exceeding a threshold of 400,000 cells/ml and observed no differences in milk production and SCC between treated and untreated control cows. The majority (58%) of the cost of this program was due to the discarded milk associated with treatment, representing a net loss which was presumed to be increased due to treatment of 49 (48%) culture negative cows. Recent economic models of lactation therapy of subclinical mastitis have included bacteriologic culture results as a criterion for treatment and have suggested treatment be limited to chronic IMI to avoid treatment of transient infections that spontaneously cure (Swinkels et al., 2005a; Swinkels et al., 2005b). These studies have suggested that targeted therapy of chronic subclincal mastitis
caused by *S. aureus*, *S. uberis*, or *S. dysgalactiae* may be beneficial under some scenarios, especially where contagious transmission risk is high and the benefit of treatment includes reduced costs by preventing new infections (Swinkels et al., 2005a; Swinkels et al., 2005b).

*Further rationale for subclinical mastitis control: Indirect effects of therapy*

Cases of subclinical mastitis caused by major gram positive pathogens may constitute a reservoir of bacteria that are an important source of infection for other cattle in a herd (Dodd et al., 1969; Bramley and Dodd, 1984; Zadoks et al., 2002a; White et al., 2006). Hence, successful bacteriologic cure of subclinical mastitis has a direct effect on the individual animal, but may also have an indirect effect on other members of a herd, in that it reduces exposure to pathogenic organisms. A number of authors have identified strategies to control subclinical mastitis on dairy farms, and many of these strategies target treatment of chronic subclinical mastitis in individual quarters or cows (Dodd et al., 1969; Bramley and Dodd, 1984; Hillerton and Berry, 2003; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005; Swinkels et al., 2005a). As described in a previous section, the efficacy of lactation therapy for treatment of individual cows with subclinical mastitis has been demonstrated in both experimental challenge studies and clinical field trials (Sol et al., 1997; Gillespie et al., 2002; Oliver et al., 2003; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005), and may be referred to as the direct effect of therapy which is most commonly measured in individual level study designs (Halloran and Struchiner, 1991; Hayes et al., 2000; Farrington, 2003). However, the
incidence of disease for pathogens which may be transmitted between hosts may depend on the prevalence of disease in a population (Halloran and Struchiner, 1991). Therefore, interventions that target control of contagious or transmissible infectious disease may have indirect or population level effects (Halloran et al., 1997; Hayes et al., 2000; Farrington, 2003). In dairy herds these indirect effects of mastitis treatment strategies may include the reduced risk of pathogen transmission in the population following bacteriologic cure of infected individuals (Zadoks et al., 2002a; Swinkels et al., 2005a; Swinkels et al., 2005b). However, no field trials have evaluated the potential population level effects of lactation therapy for subclinical mastitis caused by the major gram-positive pathogens other than *Streptococcus agalactiae*. One reason for this lack of research may be that study designs that assess indirect effects of interventions on mastitis transmission dynamics are labor intensive and expensive. Another reason may be that the presumed variability in pathogen transmission dynamics across farms would require that large numbers of farms be enrolled in multi-location field trials to evaluate the impact of interventions under a variety of management conditions.

*Evaluating interventions at the population level: Use of deterministic models*

As early as 1969, Dodd and others recognized the importance of population level measures and mathematical models in evaluating mastitis control strategies (Dodd et al., 1969). However, it has only been recently that a small number of studies have incorporated population level mathematical models to describe the dynamics of pathogen transmission and the overall effects of interventions such as post milking teat disinfection
or other control strategies (Lam et al., 1996; Allore and Erb, 1999; Zadoks et al., 2002a; White et al., 2006). The use of deterministic state-transition compartmental models to describe pathogen transmission dynamics (SEIR models, where S, E, I, and R represent the infection status compartments susceptible, latent, infectious, and recovered, respectively) allows for the estimation of population level measures such as the basic reproductive number ($R_0$) for a pathogen (Anderson and May, 1991; Lam et al., 1996; Zadoks et al., 2002a). Essentially, $R_0$ describes the tendency of a pathogen to spread in a population of susceptible hosts, and is defined as the average number of secondary infections resulting from the introduction of one infectious individual into a fully susceptible population (Anderson and May, 1991; Vynnycky and Fine, 1998). $R_0$ describes the tendency of a pathogen to spread in a population of hosts, and is a function of the probability per unit time that one infectious individual will infect a susceptible individual (the transmission parameter, $\beta$), and of the duration of infection. The transmission parameter, $\beta$, is the coefficient that relates the transmission rate and type of transmission function to the frequencies or densities of infectious and susceptible hosts in a population (Anderson and May, 1991; Lam et al., 1996; McCallum et al., 2001). An effective or net reproductive number ($R_t$) can be determined as the expected number of secondary cases per infectious case where a fraction of the population is susceptible, or in other words, as the number of secondary infections arising from each infectious case in a given population (Anderson and May, 1991; Vynnycky and Fine, 1998; Zadoks et al., 2002a). $R_0$ and $R_t$ are useful summary measures to examine the potential impact of control programs or to compare the transmission potential of pathogens in different
populations having different epidemiologic conditions (Lam et al., 1996; Cherry et al., 1998; Vynnycky and Fine, 1998; Zadoks et al., 2002a; White et al., 2006). In summary, a primary advantage of SEIR models is they incorporate the effect of population level variables including numbers of susceptible and infectious individuals and thus these models have use in quantifying the overall impact of interventions such as vaccination, quarantine or removal, or antibiotic treatment programs (Cherry et al., 1998; Longini et al., 1998; Bonten et al., 2001; Pourbohloul et al., 2003). The use of quantitative epidemiologic methods that rely on cow-level data to estimate group or population level parameters of pathogen transmission offer advantages over previous methods of analysis as they incorporate the dependent effect of population-level pathogen prevalence and the transmission parameters of pathogens (Lam et al., 1996). The collection of data from a longitudinal field trial in a population of dairy cattle managed under typical commercial conditions provides the opportunity to test relevant herd level clinical outcomes associated with implementation of a mastitis control program (Zadoks et al., 2002a). While antibiotic treatment is a common practice implemented in mastitis control, few studies have applied mathematical models of pathogen transmission to estimate the efficacy of antibiotic based mastitis control programs.

*Evaluating interventions at the molecular level: Use of molecular epidemiology in field trials*

Zadoks and Schukken (2006) have reviewed the value and use of molecular strain typing in the evaluation of transmission dynamics of veterinary pathogens. The potential
significance of strain typing in mastitis control research has also been described
(Barkema et al., 2006). As indicated in these reviews, identification of strain types within
and between farms may provide evidence of associations between strain type and
infection outcome. For example, Haveri et al. (2005) and Zadoks et al. (2000; 2003) have
described associations between bacterial genotype and infection severity and duration for
*S. aureus* and *S. uberis* mastitis. In some cases these associations have been expanded to
include identification of potential virulence genes that may be linked to *S. aureus* strain
types causing specific clinical manifestations in bovine mastitis (Haveri et al., 2007). In
the broader context of understanding host specificity and strain adaptation, recent studies
have characterized genes unique to *S. aureus* isolates recovered from cases of bovine
mastitis, providing genetic evidence of host specialization and virulence factors of *S.
aureus* clonal groups associated with cattle (Herron-Olson et al., 2007). One study
discovered that the reference *S. aureus* strain Newbould 305 (NCIMB 702892) frequently
used in experimental challenge and in vitro experiments may be more closely associated
with cattle skin isolates than naturally occurring intramammary isolates (Smith et al.,
2005b), although this finding may not be a great surprise to the researchers who
pioneered use of this strain, as a less virulent strain has significant advantages in
experimental challenge models and this strain may have been originally selected based on
its less virulent phenotype. A number of recent studies utilizing molecular typing
methods [e.g. (Haveri et al., 2007; Herron-Olson et al., 2007)] have corroborated the
potential importance of some previously recognized host and virulence factors associated
with bovine *S. aureus* mastitis (Devriese, 1984; Bramley et al., 1989). For example,
bovine ecovars appear to be staphylokinase negative and beta-hemolysin (Hlb) positive, while staphylokinase positive and Hlb positive *S. aureus* strains are primarily human-specific ecovars (Devriese, 1984). Recent work has demonstrated that the Hlb-converting bacteriophages, which integrate specifically in the Hlb gene, and upon integration convert isolates to an Hlb-negative phenotype, carry a cluster of genes associated with innate immune system invasion and modulation in humans (Goerke et al., 2006; van Wamel et al., 2006). These phage mediated virulence factors appear to be positively selected for and are more frequently found among human isolates associated with chronic infections (e.g. lung infections in cystic fibrosis patients and cases of bacteremia) versus nose isolates colonizing healthy individuals. An analogous situation may be identified among bovine isolates, where isolates associated with skin colonization appear to be distinct from those associated with mastitis (Zadoks et al., 2002b; Smith et al., 2005b). These examples illustrate how molecular methods may be used in an epidemiologic context to provide a finer level of detail in our understanding of disease processes in populations. On the purpose of molecular epidemiology, Bruce Levin et al. (1999) provide this summary, “The practical goals of molecular epidemiology are to identify the microparasites (viruses, bacteria, fungi, and protozoa) responsible for infectious diseases and determine their physical sources, their biological (phylogenetic) relationships, and their routes of transmission and those of the genes (and accessory elements) responsible for their virulence, vaccine-relevant antigens, and drug resistance.” These authors consider molecular epidemiology the logical adjunct (or perhaps descendant) to earlier typing schemes (such as serologic, phenotypic, or phage typing).
which were applied to understand “variation in the incidence and severity of infections with microbes classified as members of the same species” (Levin et al., 1999).

In the context of intervention studies, such as mastitis treatment efficacy trials, molecular strain typing has been proposed as a method to refine our understanding of transmission dynamics at the farm or treatment group level and of pathogen cure and reinfection rates (Barkema et al., 2006; Zadoks and Schukken, 2006).

Zadoks et al. (Zadoks et al., 2001a) and Phuektes et al. (Phuektes et al., 2001b) provide clear examples of the use of molecular epidemiologic methods that provided fresh insight to the potential of cow to cow transmission of *S. uberis* mastitis within dairy herds. This has led Zadoks to propose a conceptual framework for *S. uberis* epidemiology which hypothesizes that strains of this species might be separated into host-adapted and non-host-adapted ecotypes (Zadoks, 2007). Characteristics of the host-adapted ecotype were proposed to include low strain heterogeneity and subclinical presentations with a long duration of infection. A contagious source of exposure would dominate for the host-adapted ecotype leading to a higher risk of infection during lactation. In comparison, characteristics of a non-host-adapted ecotype were suggested to include high strain heterogeneity and a short duration of infection more frequently with clinical manifestations. The source of exposure for the non-host adapted ecotype would be the environment, leading to new infections in both lactating and non-lactating cattle (Zadoks, 2007). Based on evidence of host specificity for *S. aureus* (Devriese, 1984; Zadoks et al., 2000; Zadoks et al., 2002b; Smith et al., 2005b; Herron-Olson et al., 2007),
a similar ecotype hypothesis seems appropriate for this species, and may be expanded to
include bovine host-adapted, human host-adapted, and non-host adapted ecotypes.

In a practical application, it has been suggested that molecular typing can be used
in conjunction with epidemiologic and clinical data to differentiate between contagious
and environmental sources of exposure for pathogens causing bovine mastitis (Zadoks
and Schukken, 2006; Zadoks, 2007). The finding of multiple bacterial strains of a single
species causing mastitis in a population of cows suggests these infections arise from one
or more sources of exposure that are populated with many strains (e.g. the environment).
Such findings have been used to infer environmental sources of exposure for *S. uberis*
mastitis in dairy herds where the environment is populated with a diverse number of
strain types (Wieliczko et al., 2002; Zadoks et al., 2003; Pullinger et al., 2006; Pullinger
et al., 2007). In comparison, the finding of a limited number of strains within a herd
experiencing a mastitis outbreak over a defined time period may be consistent with cow
to cow transmission as has also been suggested for *S. uberis* in some herds or
management scenarios (Phuektes et al., 2001b; Zadoks et al., 2001a). In these later cases
it is possible that clusters of infection with a limited number of strains in space or time
can also arise from a common environmental source populated with a dominant strain, or
from the enhanced virulence of some individual environmental strains (Phuektes et al.,
2001b). Because indirect effects of mastitis treatment strategies may include the reduced
risk of pathogen transmission in the population following bacteriologic cure of infected
individuals, the ability to demonstrate these effects is likely driven by the contagious
transmission potential of individual strains of a pathogen within a herd. Therefore it
follows that identification of indirect effects of a subclinical mastitis treatment program would be improved by characterization of strain diversity for the targeted species of pathogens in a clinical field trial.

The ability to differentiate strains by molecular typing may also improve estimates of infection duration and the proportion of bacteriologic cure in studies of direct effects of mastitis therapy (Luby and Middleton, 2005; Barkema et al., 2006; Zadoks and Schukken, 2006). Few veterinary intervention studies appear to have incorporated molecular strain typing methods in studies of treatment efficacy, although the importance of including molecular genotyping to distinguish recrudescent and new *Plasmodium falciparum* infections following treatment has been described for human anti-malarial trials (Mugittu et al., 2007).

Luby and Middleton (2005) provided improved estimates of cure proportions by strain typing pre-treatment and post-treatment isolates for infected quarters that did not appear to cure. These authors identified one of 10 chronic *S. aureus* infections where the post-treatment isolate differed from the pre-treatment isolate in DNA digest pattern on pulsed-field gel electrophoresis (PFGE). They used this data to define this quarter as cured of the first strain type and reinfected with a different strain type. Other authors have suggested that some differences in the PFGE fingerprint identified during chronic *S. aureus* infections in cystic fibrosis patients are due to genomic alterations of a single infecting strain over time (Goerke et al., 2004). Interestingly, the extent of DNA digest banding pattern differences observed in these two studies appears to be similar. In both publications there appear to be one to three identifiable differences in fragment patterns
suggesting the isolates are closely related and consistent with a single genetic event (i.e. a mutation, insertion, or deletion) (Tenover et al., 1995). Goerke et al. (Goerke et al., 2006) identified shifts in band sizes among \textit{S. aureus} isolates from chronically infected cystic fibrosis patients that were attributed to phage mobilization, and these authors suggested the changes were a result of selective pressure on the pathogen due to the host response or regular antibiotic exposure during chronic infections, but not associated with a new infection caused by a different strain. In contrast, Luby and Middleton (Luby and Middleton, 2005), suggested that what appeared to be a single change in band size was attributed to a infection caused by a different strain. These authors did not address the possibility that the difference in PFGE pattern observed over the course of this infection could be attributed to a genetic change in the infection strain type in response to treatment. Additional research appears warranted to evaluate the extent of genetic variation in chronic \textit{S. aureus} bovine mastitis cases associated with treatment or host responses, or the frequency of potential post treatment reinfections that may lead to underestimation of cure rates associated with therapy.

\textit{Molecular methods of bacterial strain typing}

The extensive number of molecular typing methods available for discriminating among bacterial strain types is beyond the scope of this review, and has been briefly addressed by other authors (Zadoks and Schukken, 2006), and more extensively reviewed elsewhere (Maslow et al., 1993; Riley, 2004).

24
PFGE and multilocus sequence typing (MLST) are two molecular typing methods that meet the performance criteria described by Maslow (1993) for discriminating among *S. aureus* isolates, including those obtained from dairy cattle milk, skin and the farm environment. Although other methods may have some advantages such as reduced costs or labor, both PFGE and MLST have been demonstrated to be highly suitable for epidemiologic analysis of bovine *S. aureus* isolates (Fitzgerald et al., 1997; Zadoks et al., 2000; Zadoks et al., 2002b; Sabour et al., 2004; Jorgensen et al., 2005; Smith et al., 2005a; Smith et al., 2005b; Anderson and Lyman, 2006; Anderson et al., 2006; Dingwell et al., 2006; Aires-de-Sousa et al., 2007; Haveri et al., 2007; Rabello et al., 2007). Comparison of the two methods appears favorable, with strong concordance of results for bovine *S. aureus* isolates, although PFGE may provide a higher level of discrimination and thus may be more appropriate for investigations conducted within herds (Jorgensen et al., 2005; Smith et al., 2005b; Aires-de-Sousa et al., 2007).

Random amplified polymorphic DNA (RAPD) appears to have good discriminatory power for studies of *S. uberis* epidemiology within dairy herds (Gillespie et al., 1998; Oliver et al., 1998; Wieliczko et al., 2002; Zadoks et al., 2003). The identification and typing of streptococcal species may prove to be more of a challenge given the high level of recombination found in this group (Lefebure and Stanhope, 2007), and *S. uberis* has been described as having a high extent of recombination compared to the other streptococcal pathogens (Stanhope personal communication, July, 2008).
Outline of this Dissertation

Following this general introduction (Chapter 1), the theoretical basis of population level effects of lactation therapy is explored through development of a deterministic mathematical model in Chapter 2. In this chapter, the potential indirect and overall effects of treatment of chronic cases of subclinical mastitis on the dynamics of mastitis transmission for pathogens that can spread from cow to cow are estimated under a range of transmission scenarios. In Chapter 3, the hypothesis that treatment of chronic subclinical *Staphylococcus aureus* mastitis in a population of dairy cattle results in the reduction of duration of *S. aureus* infection (direct effect of therapy) and reduction of incidence of new *S. aureus* intramammary infection (indirect effect of therapy) is tested. Pulsed field gel electrophoresis (PFGE) is used to assess the effect of the treatment program on strain specific dynamics of *S. aureus* infection. In addition the effect of the treatment program on somatic cell count following treatment is described. In Chapter 4, the direct and indirect effects of treating chronic subclinical mastitis caused by streptococcal species are explored, with a focus on treatment of chronic subclinical *Streptococcus uberis* infections. In this chapter, the molecular epidemiology of *S. uberis* infections is examined for two herds using random amplified polymorphic DNA (RAPD) methods. In addition, the association between response to therapy and pretreatment antimicrobial resistance phenotypes is described. Chapter 5 introduces an unexpected finding that emerged from these studies, by examining the association between *Coxiella burnetti* shedding in milk and subclinical mastitis. These findings are used in Chapter 6, to support conclusions regarding the the potential benefits and limitations of lactation
therapy for subclinical mastitis. In Chapter 6 additional data on strain typing *S. aureus*
using multilocus sequence type (MLST) methods is presented and compared to results of
PFGE and antimicrobial susceptibility typing. Finally, Chapter 6 provides suggestions
for future research in subclinical mastitis control.
A mathematical model demonstrating indirect and overall effects of lactation therapy targeting subclinical mastitis in dairy herds

John W. Barlow\textsuperscript{1}, Lisa J White\textsuperscript{3}, Ruth N. Zadoks\textsuperscript{2}, and Ynte H. Schukken\textsuperscript{2}

\textsuperscript{1}Department of Animal Science, University of Vermont, 204 Terrill Hall, 570 Main Street, Burlington, VT 05405, USA.

\textsuperscript{2}Quality Milk Production Services, Department of Population Medicine and Diagnostic Sciences, Cornell University, 22 Thornwood Drive, Ithaca, NY 14850-1263, USA.

\textsuperscript{3}Ecology and Epidemiology Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK.

Accepted

Preventive Veterinary Medicine
Abstract

A deterministic state-transition model for mastitis transmission was developed to explore population level effects of antibiotic treatment regimens targeting chronic subclinical mastitis caused by major gram-positive pathogens in lactating dairy cows. Behavior and sensitivity of model outputs to changes in key parameters were explored. Outcomes included the size of the state variables describing proportions of infected quarters and basic and effective reproductive numbers. Treatment effects were estimated by calculating proportional reductions in state variables at equilibrium for populations implementing a treatment program relative to populations with no intervention. In general the relationships between parameters were complex and non-linear, although the model was especially sensitive to changes in the value of the transmission rate parameter. Interaction between the parameters resulted in large variations in treatment effect estimates. Effect estimates calculated from model outputs showed a quadratic curve with a clear optimum at low, but not the lowest, transmission rates. These results indicate that overall positive population level effects of lactation therapy would be realized for herds that have successfully implemented practices that reduce the transmission rate of pathogens. A key finding is that in herds with high transmission rates, treatment of chronically infected quarters was predicted to have little impact on the proportion of infected quarters and no positive population level effect in reducing the force of infection and new infection rates. Results of this study suggest that field trials to evaluate efficacy of antimicrobial treatment should include estimates of indirect treatment effects.
Subclinical mastitis is the dominant form of mastitis affecting dairy cattle yet often goes undetected or is left untreated (Bramley and Dodd, 1984; Hillerton and Berry, 2003; Oliver et al., 2004). Cases of subclinical mastitis caused by major gram positive pathogens constitute a reservoir of bacteria that are an important source of infection for other cattle in a herd (Dodd et al., 1969; Bramley and Dodd, 1984; Zadoks et al., 2002a; White et al., 2006). Subclinical mastitis may be of short duration (transient infections which spontaneously cure) or extended duration (chronic or persistent infections of more than one or two months) (Zadoks et al., 2003; Swinkels et al., 2005a; Swinkels et al., 2005b). A number of authors have identified strategies to control subclinical mastitis on dairy farms, including antibiotic treatment of chronic cases during lactation (Dodd et al., 1969; Bramley and Dodd, 1984; St Rose et al., 2003; Zadoks et al., 2003; Oliver et al., 2004; Swinkels et al., 2005a; Swinkels et al., 2005b). The direct effect of lactation therapy for subclinical mastitis has been described in both experimental challenge studies and clinical field trials (Gillespie et al., 2002; Oliver et al., 2003; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005). Interventions targeting control of infectious disease may also have indirect or population level effects as incidence may depend on prevalence of disease in a population (Halloran et al., 1997; Hayes et al., 2000; Farrington, 2003). In dairy herds the indirect effects of mastitis treatment strategies may include the reduced risk of pathogen transmission in the population following
bacteriologic cure of infected individuals (Zadoks et al., 2002a; Swinkels et al., 2005a; White et al., 2006).

As early as 1969, Dodd and others recognized the importance of population level measures and mathematical models in evaluating mastitis control strategies (Dodd et al., 1969). However, it has only been recently that a small number of studies have used population level mathematical models to describe the dynamics of mastitis transmission and the overall effects of interventions (Lam et al., 1996; Allore and Erb, 1999; Zadoks et al., 2002a; White et al., 2006). The use of deterministic state-transition compartmental models to describe pathogen transmission dynamics (SEIR models, where S, E, I, and R represent the infection status compartments susceptible, latent, infectious, and recovered, respectively) allows for the estimation of population level measures such as the basic reproductive number (R\(_0\)) (Anderson and May, 1991; Lam et al., 1996; Zadoks et al., 2002a). Essentially, R\(_0\) describes the tendency of a pathogen to spread in a population of susceptible hosts, and is defined as the average number of secondary infections resulting from the introduction of one infectious individual into a fully susceptible population (Anderson and May, 1991; Diekmann and Heesterbeek, 2000). An effective or net reproductive number (R\(_t\)) can be determined as the expected number of secondary cases per infectious case where only a fraction of the population is susceptible (Anderson and May, 1991; Diekmann and Heesterbeek, 2000). R\(_0\) and R\(_t\) are useful summary measures to examine the potential impact of control programs or to compare the transmission potential of pathogens in different populations under different epidemiologic conditions (Lam et al., 1996; Cherry et al., 1998; Vynnycky and Fine, 1998; Zadoks et al., 2002a;
White et al., 2006). SEIR models incorporate population level variables in predicting disease transmission dynamics over time, and these models have been used to quantify the overall impact of interventions such as vaccination, quarantine or removal, or antibiotic treatment programs (Cherry et al., 1998; Bonten et al., 2001; Longini et al., 2002; Pourbohloul et al., 2003).

The primary objective of this research was to explore the potential effects of antibiotic treatment regimens targeting chronic subclinical mastitis caused by major gram-positive pathogens. The main effect of interest was the indirect effect of treating chronic intramammary infections during lactation on new infection rates and the force of infection. We describe the development and use of a deterministic state-transition model for subclinical mastitis transmission dynamics to quantify indirect effects of a targeted intervention. The impact of parameter estimate variation and the interaction between parameter estimates, including the transmission and cure rate estimates, on direct, indirect, and overall effects of lactation therapy was explored.

Methods

Model development

The model is a variant of the classic infectious disease epidemic models (SEIR) described by Anderson and May (1991), with modifications specific to mastitis transmission in dairy herds to account for 1) the apparent absence of a latent period, and 2) recurrent infection due to the apparent absence of, or very short duration of, immunity
The present model was developed for a population of lactating udder quarters divided into four states, susceptible (S), newly infected (IN), chronically infected (IC), and recovered-susceptible (RS), where the compartments represent the proportion of lactating quarters in the population in each state. The dynamics of state transitions are illustrated in figure 2.1.

Figure 2.1 Flow diagram of the state-transition model of subclinical mastitis transmission for estimation of the impact of mastitis treatment strategies targeting chronic subclinical mastitis in a population of lactating quarters of dairy cattle. The boxes represent the state variables and arrows represent the flow rates between naïve susceptible (S), newly infected (IN), chronically infected (IC), and recovered susceptible (RS) states. Lettering represents the variables and parameters in the mathematical model (equations [1 – 14]; table1). The superscript i represents the cure rate associated with populations that differ in application of a mastitis treatment strategy, where i=1 for herds implementing an intervention and i =0 for herds not implementing a subclinical mastitis treatment strategy (see table 2.1 for default values).

The model was structured on the assumption that IMI status is strictly defined by serial bacteriologic culture results where the sample interval is approximately 1 month using (Lam et al., 1996; Zadoks et al., 2002a; White et al., 2006).
previously published definitions of infection (Hogan, 1990; Dohoo, 1991; Lam et al., 1997b; Zadoks et al., 2001a). The model was defined by a set of four ordinary differential equations describing the change in proportion of quarters in each state over time (equations [2.1] – [2.4]),

\[
\frac{dS}{dt} = b\theta_s - \lambda_s S - \mu S \\
\frac{dI_N}{dt} = b\theta_N + \lambda_s S + \lambda_R R_N - (\mu + \alpha_N)I_N - (1 - \rho)v_N I_N - \rho v_N I_N \\
\frac{dI_C}{dt} = b\theta_C + \rho v_N I_N - (\mu + \alpha_C)I_C - v_C I_C \\
\frac{dR_S}{dt} = b\theta_R + v_C I_C + (1 - \rho)v_N I_N - \lambda_R R_S - (\mu + \alpha_R)R_S
\]

where

\[
N = S + I_N + I_C + R_S = 1 \\
\theta_s = 1 - (\theta_N + \theta_C + \theta_R) \\
\lambda_s = \beta_s (I_N + \epsilon I_C) \\
\lambda_R = \beta_R (I_N + \epsilon I_C) \\
b = \mu N + \alpha_N I_N + \alpha_C I_C + \alpha_R R_S \\
\beta_R = \delta \beta_s \\
v_N = \frac{1}{\hat{d}_N} \\
v_C = \frac{1}{\hat{d}_C}
\]

with the meaning of all symbols defined in table 2.1.
Table 2.1 Definitions of variables and initial parameter estimates for the subclinical mastitis model in a population of udder quarters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Units</th>
<th>Definition</th>
<th>Estimate ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S$</td>
<td>Normalized</td>
<td>Proportion of lactating population with no previous evidence of IMI (naïve susceptible)</td>
<td>initial = 0.90</td>
</tr>
<tr>
<td>$I_N$</td>
<td>Normalized</td>
<td>Proportion of lactating population with new subclinical IMI</td>
<td>initial = 0.0075</td>
</tr>
<tr>
<td>$I_C$</td>
<td>Normalized</td>
<td>Proportion of lactating population with chronic subclinical IMI</td>
<td>initial = 0.0425</td>
</tr>
<tr>
<td>$R_S$</td>
<td>Normalized</td>
<td>Proportion of lactating population not currently infected with history of previous IMI (recovered)</td>
<td>initial = 0.05</td>
</tr>
<tr>
<td>$\lambda_S$</td>
<td>day⁻¹</td>
<td>Force of infection – coefficient of transmission between I and S individuals</td>
<td></td>
</tr>
<tr>
<td>$\lambda_R$</td>
<td>day⁻¹</td>
<td>Force of infection – coefficient of transmission between I and R individuals</td>
<td></td>
</tr>
<tr>
<td><strong>Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\mu$</td>
<td>day⁻¹</td>
<td>Rate of turnover of quarters (cull, death, dry-off)</td>
<td>0.0040</td>
</tr>
<tr>
<td>$\alpha_i$</td>
<td>day⁻¹</td>
<td>Additional rate of turnover of quarters in $I_N$, $I_C$, or $R_S$</td>
<td>$\alpha_N$ = 0.00401, $\alpha_C$ = 0.00261, $\alpha_R$ = 0.0009</td>
</tr>
<tr>
<td>$\delta$</td>
<td>-</td>
<td>Transmission rate coefficient for increased risk of previously infected quarter (see text)</td>
<td>3</td>
</tr>
<tr>
<td>$d_{I_N}$</td>
<td>day</td>
<td>Duration estimate in $I_N$ state</td>
<td>$\hat{d}_{I_N}$ = 30 days</td>
</tr>
<tr>
<td>$d_{I_C}$</td>
<td>day</td>
<td>Duration estimate in $I_C$ state</td>
<td>$\hat{d}_{I_C}$ = 95 days</td>
</tr>
<tr>
<td>$\rho$</td>
<td>-</td>
<td>Proportion new IMI that progress to chronic state</td>
<td>0.74</td>
</tr>
<tr>
<td>$\beta_i$</td>
<td>capita⁻¹ day⁻¹</td>
<td>Transmission rate</td>
<td>$\beta_S$ = $\delta \beta_S$ = 0.024</td>
</tr>
<tr>
<td>$\nu_i$</td>
<td>day⁻¹</td>
<td>Recovery or cure rate</td>
<td>$\nu_N$ = 0.03333, $\nu_C$ = 0.010526, $\nu_C'$ = 0.025³</td>
</tr>
<tr>
<td>$\theta_i$</td>
<td>Normalized</td>
<td>Proportion of individuals entering the lactating herd</td>
<td>$\theta_N$ = 0.0075, $\theta_C$ = 0.0425, $\theta_R$ = 0.05</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>-</td>
<td>Coefficient that reflects potential difference in transmissibility between new IMI and chronic IMI</td>
<td>$\epsilon_N$ = 1 - (1 - $\theta_N$ + $\theta_C$ + $\theta_R$) = 0.90</td>
</tr>
</tbody>
</table>

1. Initial variable estimates based on field observations from referenced studies with per capita rates expressed as events per 10⁷ quarter days at risk. References for source of estimates are described in methods section of text.
2. Calculated from equation 11 where it was assumed the mean duration in the $I_N$ state is 30 days.
3. Calculated from equation 12 where it was assumed the mean duration in the $I_C$ state is 95 days in herds not implementing treatment of subclinical mastitis during lactation, and 40 days in herds implementing diagnosis and treatment of subclinical mastitis.
The total population size \((N)\) was assumed to be constant, set to unity, and equals the sum of the proportion of quarters in each state \([2.5]\). Therefore all exits from the lactating herd were replaced by entries. Exits from the population were represented by the rate parameters \(\mu\) and \(\alpha_i\), where \(\mu\) represents the general exit rate for any reason other than mastitis (including dry-off), and \(\alpha_i\) represents additional exit rates from the infected and recovered states. Quarters were assumed to enter the population into all states at a rate equivalent to the sum of all exit rates \((b)\) \([2.9]\), where \(\theta_i\) represents the proportion of quarters entering each state. Parameters \(\lambda_s\) and \(\lambda_r\) are the force-of-infection probabilities that susceptible or recovered-susceptible quarters become newly infected, respectively. Each of the force-of-infection parameters are a function of the transmission parameter \((\beta_i)\) and the total proportion of infected quarters in the population \((I_N+I_C)\) \([2.7-2.8]\). A transmissibility coefficient \((\varepsilon)\) was included in the model to account for potential differences in transmissibility between new IMI and chronic IMI quarters; however, because there is limited evidence demonstrating that chronically infected quarters are more or less likely than new infections to transmit infection upon contact with susceptible quarters this coefficient was set to 1 in all iterations of the present analysis. Additional assumptions regarding parameter estimates are described in the individual sections that follow. Realizations of the model were obtained using the computer software package Berkeley Madonna (Version 8.0.1, Macey & Oster, University of California, Berkeley, CA, 2000). Initial parameter estimates and their ranges were obtained from peer-reviewed publications, are defined in table 2.1 and briefly described below.
Entry into the lactating herd

The model was developed as a proportional model for a constant population size. This is consistent with a year-round calving system where herd size is stable. Entries into the lactating herd may come from either primiparous or multiparous cows, with all multiparous cows assumed to have received dry cow therapy. Potential heterogeneity in mastitis risk among replacement animals was ignored and entries were modeled in the simplest form, with a fixed proportion of quarters entering each state from a uniform population of replacements. These entries were represented by the parameters $\theta_S$, $\theta_N$, $\theta_C$, and $\theta_R$, for the respective states $S$, $I_N$, $I_C$, $R_S$, where $\theta_S + \theta_N + \theta_C + \theta_R = 1$. Each $\theta_i$ was then multiplied by the net replacement rate ($b$) [2.9] to replace all quarters that exit from the herd. Fixed estimates for values of influx parameters were obtained from published field trial data (Lam et al., 1997b; Zadoks et al., 2002a; Zadoks et al., 2003; White et al., 2006). These estimates were also used to set initial proportions of quarters in each state at time zero (table 2.1).

Exits from the lactating herd

Quarters were assumed to exit the lactating herd as a result of dry-off, culling, sales, or death. Following White et al. (2001b) and Zadoks et al. (2002a), the average length of lactation was assumed to be 250 days, (i.e. efflux rate was 0.004 quarters per day), which is used as the fixed estimate for the parameter $\mu$ uniformly applied to all compartments. Additional exits from the herd were represented by $a_n$, and separate rates
were recognized for each infected state ($\alpha_N$ and $\alpha_C$) and for the recovered susceptible state ($\alpha_R$). Zadoks et al. (2002a) observed additional rates of mastitis associated culling for clinical and subclinical mastitis caused by *S. aureus* to be 0.034 and 0.0021 quarters per day, and additional rates of culling among recovered quarters with a prior mastitis episode to be 0.0009 quarters per day. The present model assumed an increased rate of exit among quarters with clinical mastitis compared to subclinical IMI, and initial estimates of $\alpha_N$ and $\alpha_C$ are based on the rates observed by Zadoks et al. (2002a) while accounting for the average proportion of quarters likely to present with a clinical episode in each of the infected states. Based on data obtained from the literature the current model assumed 30% of new and 25% of chronic major gram-positive IMI display clinical signs (Dodd et al., 1969; Todhunter et al., 1995; Lam et al., 1997b; Zadoks et al., 2003; and unpublished data of R.N. Zadoks). Fixed estimates of mastitis associated exit rate from the $I_N$ state was calculated as, (‘Exit rate for clinical mastitis’ * ‘proportion of days clinical in $I_N$’ * ‘proportion showing clinical signs in $I_N$’) + (‘Exit rate for subclinical mastitis’) * (1-‘proportion of days clinical in $I_N$’) * (‘proportion showing clinical signs in $I_N$’) + (‘Exit rate for subclinical mastitis’) * (1-‘proportion showing clinical signs in $I_N$’). Similarly exit rate from the $I_C$ state was calculated as (‘Exit rate for clinical mastitis’ * ‘proportion of days clinical in $I_C$’ * ‘proportion showing clinical signs in $I_C$’) + (‘Exit rate for subclinical mastitis’) * (1-‘proportion of days clinical in $I_C$’) * (‘proportion showing clinical signs in $I_C$’) + (‘Exit rate for subclinical mastitis’) * (1-‘proportion showing clinical signs in $I_C$’). Based on original data of Lam (1996) and Zadoks (2002), we assumed the average duration of a clinical mastitis event caused by major gram-
positive pathogens to be 6 days, and the total average duration in the \( I_N \) and \( I_C \) states to be 30 and 95 days respectively. Therefore \( \alpha_N = (0.034 * 6/30 * 0.30) + (0.0021 * 24/30 * 0.30) + (0.0021 * 0.70) = 0.00401 \), and \( \alpha_C = (0.034 * 6/95 * 0.25) + (0.0021 * 89/95 * 0.25) + (0.0021 * 0.75) = 0.00261 \) (table 2.1).

New infection rates

Initial values for the transmission parameters were obtained from review of the literature, and were influenced by the observations of Zadoks et al. (2001a; 2002a) that transmission parameters for new infections from the naïve susceptible state are lower than those from the recovered state. This difference has been attributed to increased susceptibility of recovered susceptible quarters compared to naïve uninfected quarters, and is in agreement with observational epidemiologic studies (Zadoks et al., 2001b). This epidemiologic study showed that increased susceptibility of recovered susceptible quarters is not species specific, i.e. recovery from \( S. aureus \) and from \( S. uberis \) were both associated with increased risk of subsequent new infection with either pathogen (Zadoks et al., 2001b). This implies that the increased risk of infection in recovered susceptible quarters is not due to undiagnosed chronic infections with episodes of very low bacterial shedding. In the 3 herds studied by Zadoks et al. (2002a) the estimated transmission parameters for new \( S. aureus \) infections from \( R_S \) were between 3 and 6 times greater than those for new infections from the susceptible state. Similarly during a \( S. uberis \) outbreak in one of these herds, the estimated transmission parameter for new infections among recovered susceptible quarters was approximately 7.5 times higher than naïve susceptible
quarters (Zadoks et al., 2001a). The current model structure allows input of a baseline transmission parameter for the susceptible state ($\beta_S$), with $\beta_R$ expressed as a function of $\beta_S$ multiplied by an ‘inflation factor’ ($\delta$) [2.10], with $\delta$ set to a value between 1 and 8. In general, estimates of $\beta$ for mastitis pathogens have been obtained from Poisson regression models of new infection incidence from longitudinal studies using data describing the change over time in the size of infected, susceptible and total population size (Lam et al., 1996; Zadoks et al., 2001a) or from time series data fit to a mathematical transmission model (White et al., 2006). Detailed longitudinal studies of subclinical mastitis incidence are labor intensive and infrequent, limiting the ability to obtain pathogen specific estimates of $\beta$, $\beta_S$, and $\beta_R$ to a small number of publications (Lam et al., 1996; White et al., 2001a; Zadoks et al., 2001a; White et al., 2006). These few publications provide pathogen specific estimates of $\beta_S$ for *Staphylococcus aureus* transmission ranging from 0.0028 under steady state conditions among teats with the use of post-milking teat disinfection (PMTD), to 0.046 during a *S. aureus* outbreak in the absence of PMTD where incorrect milking machine function was also suggested to play a role in transmission (Lam et al., 1996; figure 2.2). Estimates of $\beta$ for *S. uberis* from a single herd were within the range of reported values for *S. aureus* (Zadoks et al., 2001a), although additional studies on *S. uberis* transmission dynamics are needed. Most recently, White et al. (2006), using data obtained from the longitudinal studies of seven herds (Lam et al., 1997b), reported herd level summary estimates of $\beta$ for major gram-positive pathogens with or without PMTD, ranging from 0 to 0.089 (figure 2.2). While numerous factors likely contribute to the variation observed for transmission parameter
estimates, analysis of the data from this series of references suggests that the distribution of $\beta$ estimates from 10 different herds may be bimodal, with a lower range of estimates for herds using PMTD, compared to no PMTD use (figure 2.2). From these data the mean (std. dev.) and median values of $\beta$ were 0.00868 (0.000041) and 0.00684 for populations of quarters using PMTD and 0.0362 (0.00061) and 0.0295 for populations of quarters not using PMTD. The present model was initially formulated assuming PMTD use with values for $\beta_S$ and $\delta$ set at 0.008 and 3 (thus $\beta_R = 0.024$).

![Figure 2.2: Frequency distribution of transmission parameter ($\beta_s$) estimates for major gram-positive mastitis pathogens, stratified by post milking teat disinfection (PMTD) conditions. Data from ten herds as estimated in Zadoks et al. (2001a and 2002) and White et al. (2006).](image)

Figure 2.2 Frequency distribution of transmission parameter ($\beta_s$) estimates for major gram-positive mastitis pathogens, stratified by post milking teat disinfection (PMTD) conditions. Data from ten herds as estimated in Zadoks et al. (2001a and 2002) and White et al. (2006).
Spontaneous cure rate of subclinical IMI

Estimates for spontaneous cure rates are a function of the surveillance scheme and criteria for defining a chronic IMI. In this model we assume that all new subclinical infections are observed as a single positive observation among sequential monthly observations. Therefore, assuming 30 day sampling intervals, the theoretical minimum and maximum duration in the $I_N$ compartment were assumed to be 1 and 59 days respectively, and the mean duration of new IMI ($\hat{d}_N$) in the $I_N$ state was assumed to be 30 days.

Six references from five longitudinal studies were identified that provided numeric estimates of the proportion of new IMI caused by major gram-positive pathogens that persist more than 30 days (table 2.2). Taking the numeric average of the five studies that reported specific results for major gram-positive pathogens, initial estimates assumed 74% of new IMI become chronic ($\rho=0.74$), with $1-\rho$ new IMI transitioning to the

<table>
<thead>
<tr>
<th>Species</th>
<th>Reported fraction of IMI surviving $&gt; 30$ days</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘major pathogens’</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td><em>Streptococcus spp.</em></td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

1. C = new IMI at calving, L = new IMI in lactation, O = overall
2. Authors did not stratify duration estimates by time in lactation IMI first diagnosed
3. Seven years of data from a single herd, including IMI caused by *Enterococcus* spp.
4. Author used data of Todd et al. (1995)

that reported specific results for major gram-positive pathogens, initial estimates assumed 74% of new IMI become chronic ($\rho=0.74$), with $1-\rho$ new IMI transitioning to the
recovered-susceptible state. This initial value of $\rho$ is slightly less than the 78% persistent subclinical $S.\ aureus$ IMI assumed by Swinkels et al. (Swinkels et al., 2005a) in one economic model, and slightly greater than the 70.7% proportion of $S.\ uberis$ or $S.\ dysgalactiae$ persistent IMI assumed in a separate model (Swinkels et al., 2005b).

The arithmetic mean of estimates of duration of IMI reported by Zadoks et al. (2003) and Watt (1999) for $S.\ uberis$ is 100 days, and the arithmetic mean of $S.\ aureus$ IMI duration from the reported values is 110 days (Grommers et al., 1985; Lam et al., 1996; Lam et al., 1997b; Zadoks et al., 2002a; Swinkels et al., 2005a). These estimates are similar to the mean duration of 100 days for IMI due to all major gram-positive pathogens obtained by White et al. (2006) using the data of Lam et al. (1996; 1997b). Therefore the initial duration estimate used for the present model for all major gram-positive mastitis pathogens was 100 days, and based on the assumption that 74% of IMI become chronic then the total average duration of chronic IMI was assumed to be 125 days. Given the first 30 days of a chronic IMI are in the $I_N$ state, the mean duration in the $I_C$ state is 95 days per quarter ($\hat{a}_C = 95$) and $\nu_C^0 = 0.010526$.

Cure rates associated with the treatment of chronic subclinical mastitis

Twelve reports describing the effect of antibiotic treatment regimens targeting chronic subclinical mastitis caused by major gram-positive pathogens were identified (Bramley and Dodd, 1984; Owens et al., 1997; Sol et al., 1997; Cattell et al., 2001; Gillespie et al., 2002; Hillerton and Kliem, 2002; Oliver et al., 2003; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005; Luby and Middleton, 2005; Milne et al., 2005).
Based on values from these references, reasonable estimates for cure proportions of chronic subclinical IMI caused by the major gram-positive pathogens appear to be 55, 65, and 75% for 2 day, 5 day, and 8 day treatment regimens respectively. The 75% cure proportion estimate for 8 day regimens is approximately mid-way between the estimates of 60% for *S. aureus* and 88% for *S. uberis* used by Swinkels et al. (2005a; 2005b).

Similarly, the short duration regimens approximate the cure estimate used by White et al. (2001b) for major gram-positive pathogens (60%), which these authors based on data from Sol et al. (Sol et al., 1997). For the current analysis, we assumed all chronic subclinical infections were identified by a second positive observation in sequential monthly observations. Therefore the theoretical minimum and maximum total duration of chronic IMIs at the time of diagnosis was assumed to be 30 and 60 days respectively, and the average duration of a chronic IMI at the time of diagnosis was assumed to be 45 days, with the initial 30 days occurring in the $I_N$ compartment. We assumed 6 additional days for bacteriologic culture, reporting of infection status, and initiation of treatment on the farm, thus the average duration of chronic IMIs at initiation of therapy would be a total of 51 days, with 21 days duration in the $I_C$ compartment. Based on a 75% cure proportion for an 8 day treatment regimen, the mean duration in the $I_C$ state of quarters in herds implementing an aggressive diagnosis and treatment program was calculated as $(21 \text{ days} \times 0.75) + (95 \text{ days} \times 0.25) = 40 \text{ days}$. Therefore, the initial estimate of $u_{C,I}$ in herds implementing an aggressive diagnosis and treatment program was 0.025 quarters per day. This is equivalent to reducing the total mean duration of chronic subclinical infections by 55 days as a result of the treatment program.
Estimating the effect of treatment of chronic subclinical mastitis during lactation

To explore the possible effects of treating chronic subclinical mastitis during lactation the model was numerically solved to equilibrium with parameter values in table 2.1 set as initial conditions. Following the recommendations of Halloran et al. (1991; 1997), with slight modifications, the direct, indirect, and overall effects of the intervention were explored from model outcomes. In the case of antimicrobial treatment of infected individuals in a population, the direct effect of successful treatment is to reduce duration of infection in the infected host, and the indirect effect of a treatment program is the reduction of risk of new infection among susceptible individuals in a population due to reduced infectiousness of infected hosts in the population. The direct effect of therapy in this model was strictly assumed to reduce duration of infection, and to have no effect on $\beta$. As a result, the direct effect of treatment ($DE$) was estimated as $DE = 1 - (d_1/d_0)$, where $d$ is the average total duration of chronic IMI and the subscripts 1 and 0 represent the population receiving the intervention and the population not receiving the intervention respectively. The indirect effect of the intervention in this model was assumed to be the reduction in risk of new infection. The reduced risk of infection was realized in the model output from the proportion of quarters in the new IMI state at equilibrium, and the indirect effect of treatment ($IE$) was estimated as $IE = 1 - (I_{N1}/I_{N0})$, where $I_{N1}$ and $I_{N0}$ are the steady state proportions of new IMI in populations receiving the intervention and not receiving the intervention, respectively. The overall effect ($OE$) of the intervention was defined to be the reduced prevalence of infection in populations of quarters receiving the intervention relative to populations not
receiving the intervention, and was determined from $1 - \text{the ratio of the sum of quarters in the infected states at equilibrium; } OE = 1 - \frac{(I_N + I_C)_i}{(I_N + I_C)_0}$ (Halloran et al., 1997).

**Model behavior and scenario analysis**

To explore the possible effect of treating chronic subclinical mastitis during lactation the model was numerically solved to equilibrium using the initial parameter estimates (table 1.1), for two separate values of $\nu^C_i$, where $\nu^C_i$ represented herds treating subclinical mastitis during lactation, and $\nu^C_0$ represented herds not applying diagnosis and lactation therapy of subclinical mastitis. Outcomes evaluated included the size of the 4 state variables, the sum prevalence of infected quarters ($I_T = I_N + I_C$) and uninfected quarters ($S+R_S$), the basic reproductive number ($R_0$), and the effective reproductive number ($R_t$). $R_0$ and $R_t$ were derived using previously described methods (Diekmann and Heesterbeek, 2000) and are defined as:

$$R_0 = \frac{\beta_s}{\mu + \alpha_N + v_N} + \left(\frac{\rho v_N}{\mu + \alpha_N + v_N}\right) \left(\frac{\varepsilon \beta_s}{\mu + \alpha_C + v_C}\right)$$

[2.9]

and,

$$R_t = \frac{\beta_s S + \beta_R S}{\mu + \alpha_N + v_N} + \left(\frac{\rho v_N}{\mu + \alpha_N + v_N}\right) \left(\frac{\varepsilon (\beta_s S + \beta_R S)}{\mu + \alpha_C + v_C}\right)$$

[2.10].
Simulations, sensitivity and uncertainty analysis associated with key parameters

Model behavior was explored for key parameters of interest, which were, the transmission parameter ($\beta$), transmission coefficient for increased transmission among quarters that were previously infected ($\delta$), proportion of new IMI that progress to a chronic state ($\rho$), and cure rate (quarters per day) of chronic IMI associated with treatment ($\nu_{C}^i$). The sensitivity of the model to changes in parameter estimates was examined using the ‘One At a Time’ (OAT) method of Morris (Saltelli et al., 2004). Sensitivity measures were obtained for each parameter from 10,000 iterations using randomly selected parameter values from a uniform distribution for each of the parameters, with all other parameters set at default values (table 2.1), or alternatively with the $\beta_S$ value randomly selected from a normal distribution with mean and variance as estimated from the literature for herds using PMTD or no PMTD. The values for the transmission parameter $\beta_S$, $\delta$, and $\rho$ ranged from 0 to 0.1015, 1 to 8, and 0.50 to 0.98, respectively. The values for $\nu_{C}^i$ ranged from 0.06250 to 0.00455, which are equivalent to a range of 46 to 250 days average total duration of chronic IMIs.

The impact of variability in parameter estimates on estimates of indirect and overall treatment effects was explored across the full range of potential values for the parameters $\beta_S$, $\delta$, and $\rho$. Mean model outputs were obtained from 10,000 iterations for each of 30 discrete $\beta_S$ values in 0.0035 unit steps, with $\delta$ and $\rho$ values randomly selected from uniform distributions at the start of each iteration. Using model outputs for the default values $\nu_{C}^0$ and $\nu_{C}^i$ the indirect and overall effect estimates were obtained for each discrete value of $\beta_S$. 

47
Results

For the initial default parameter values (table 2.1) the model predicted low infection prevalence at equilibrium, across the full range of $\nu_C^{i}$ values. For example, a prevalence of 6% infected quarters ($I_T=I_N+I_C$) at equilibrium was observed when $\nu_C^{i}$ was set at the hypothetical extreme of 0.00455 (i.e. the average duration of chronic IMI is set at an extreme estimate of 250 days). Comparing scenarios using the proposed default values of $\nu_C^{0}$ and $\nu_C^{i}$ (table 2.1), a reduction in the duration of chronic subclinical IMI from 125 ($\nu_C^{0}$) to 70 days ($\nu_C^{i}$) resulted in both a lower overall prevalence of infection, a lower prevalence of new IMI, as well as a reduction in both the basic and effective reproductive numbers (table 2.3 and figure 2.3). The direct effect of the treatment regimen with the higher cure rate ($\nu_C^{i}$) relative to spontaneous cure ($\nu_C^{0}$) was 0.44. The

Table 2.3  Model realization of outcome variables for fixed parameter estimates in table 2.1, for treated and control populations, where treatment reduces the mean duration of chronic subclinical infections to 70 days ($\nu_C^{i}$) compared to an untreated population where the mean duration of chronic IMI is 125days ($\nu_C^{0}$).

<table>
<thead>
<tr>
<th>Variable estimate at equilibrium</th>
<th>Duration of Chronic IMI</th>
<th>Effect estimate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125 days</td>
<td>70 days</td>
<td></td>
</tr>
<tr>
<td>$I_N$</td>
<td>0.00661</td>
<td>0.00330</td>
<td>Direct = 0.440</td>
</tr>
<tr>
<td>$I_C$</td>
<td>0.01982</td>
<td>0.00810</td>
<td>Indirect = 0.50076</td>
</tr>
<tr>
<td>Sum subclinical IMI ($I_N+I_C$)</td>
<td>0.02643</td>
<td>0.01140</td>
<td>Overall = 0.56867</td>
</tr>
<tr>
<td>$S$</td>
<td>0.88806</td>
<td>0.90421</td>
<td></td>
</tr>
<tr>
<td>$R_S$</td>
<td>0.08557</td>
<td>0.08459</td>
<td></td>
</tr>
<tr>
<td>Sum uninfected ($S + R_S$)</td>
<td>0.97357</td>
<td>0.98860</td>
<td></td>
</tr>
<tr>
<td>$R_0$</td>
<td>0.472</td>
<td>0.344</td>
<td></td>
</tr>
<tr>
<td>$R_i$</td>
<td>0.540</td>
<td>0.399</td>
<td></td>
</tr>
</tbody>
</table>
indirect and overall effects of the higher cure rate were predicted to reduce the equilibrium prevalence of new and all IMI by approximately 50.1% and 56.9%, respectively.

The model was found to be sensitive to changes in the four parameters of interest, with $\beta_5$ generally ranked as first in order of importance based on the graphical representations of the sensitivity measures as described by Saltelli et al. (2004) (figure 2.4). However, the relative impact of the parameters was dependent on the outcome measure being considered and on the fixed value of other parameters during the OAT analysis. For example, there is a larger mean and variance in outcomes associated with
variation in $\beta_S$ under conditions of low transmission (PMTD use scenarios) compared to the effect of varying other parameters across their range of values (figure 2.4a). In comparison, under high transmission conditions (no PMTD scenarios) the effect of varying other parameters on outcomes appears to be approximately equivalent to the effect of changing $\beta_S$, and the greatest variance in outcome measures is observed for changes in the duration of chronic infection parameter (figure 2.4b). Sensitivity of the model to changes in one parameter was influenced by other parameter values in a complex non-linear fashion (figure 2.5). Of particular interest were the complex interactions between $\beta_S$, $\delta$, and $\nu_C$ on the equilibrium value of $I_N$ and $R_S$ and how the size of the $R_S$ state strongly influenced rates of new infections under some scenarios. In general there was a range of values of $\beta_S$ where the model was most sensitive to changes in $\nu_C$, with changes in the duration of chronic IMI between 40 and 100 days having greatest effect on $I_N$ at $\beta_S$ near 0.014 (figure 2.5b). These results suggest that under some transmission scenarios increasing the cure rate of chronic IMI may have minimal impact on rates of new infection in a herd (figure 2.5a). In addition under conditions where $\delta > 1$ and high $\beta_S$ it appears that interventions resulting in a shorter duration of infection are associated with higher equilibrium values of $I_N$ (e.g. figure 2.5b top two lines). A key finding was that there was a non-linear relationship between changes in $\beta_S$ and model outputs demonstrating a range of $\beta_S$ values where the model was very sensitive to changes in $\beta_S$ across all other parameter combinations (figure 2.5a).
Figure 2.4  Sensitivity analysis. Steady state outcome estimates for new infection (In), chronic infection (Ic), and recovered susceptible (Rs) states obtained from model simulations. Values of $\beta_S$ were randomly sampled from a random normal distribution with mean (std dev) 0.00868 (0.000041) for populations of quarters using PMTD and 0.0362 (0.00061) for populations of quarters not using PMTD, with other parameters set at default values (table 2.1). Values of $\delta$, $\nu_C$ ($=1/\hat{d}_C$), and $\rho$ were randomly selected from uniform distributions across the full range of values, with $\beta_S$ set to mean estimates for PMTD and no PMTD scenarios. Results are mean and standard deviation (error bar) values obtained from 10,000 iterations, demonstrating increased relative sensitivity (increased variability) of the model to changes in the transmission parameter ($\beta_S$) under conditions of post milking teat disinfection (PMTD) (a.) and relative sensitivity of the model to variation in the cure rate $\nu_C$ and $\beta_S$ under conditions of no PMTD (b.) Results were similar for an alternative set of simulations where values of $\beta_S$ were randomly sampled from a uniform distribution across the full range of parameter estimates (data not shown).
Figure 2.5  a. Impact of changes in transmission parameter ($\beta_S$) on equilibrium value of new IMI ($I_N$) for two $\upsilon_C$ values, solid line (−) duration of chronic IMI = 125 days ($\upsilon_C^0 = 0.010526$), dashed line (− −) duration of chronic IMI = 70 days ($\upsilon_C^1 = 0.025$).  b. Impact of changes in $\hat{d}_C$ (=$1/\upsilon_C$), on equilibrium value of new IMI ($I_N$) for 5 $\beta_S$ values, 0.007 (−), 0.014 (····), 0.021 (──), 0.028 (− − ·), 0.035 (− − − −).
The treatment effect estimates were determined from a series of iterative model realizations across the range of parameter values. The direct effect of treatment was constant across these iterations at 0.44. The overall effect of the antibiotic therapy program targeting treatment of chronic subclinical mastitis was found to be positive across all combinations of parameters, although at high values of $\beta_S$ the overall effect estimate approached 0 regardless of the values of $\delta$ or $\rho$ (figure 2.6). The maximum indirect and overall effects were 0.88 and 0.89, respectively, and were found to occur when $\beta_S$ was 0.014, $\delta$ was in the range of 4.5 to 5.5, and $\rho$ was between 0.52 and 0.62. At these parameter values the equilibrium proportions of quarters in $I_N$, $I_C$, and $I_T$ were approximately 11, 14, and 25%, respectively in the control population compared to 1.4, 1.4, and 2.8%, in the treated population. Changes in $\rho$ across the range of values from 0.50 to 0.98 had a minimum impact on overall effect estimates for any particular combination of $\beta_S$ and $\delta$ values. In comparison, changes in $\delta$ had a large impact on overall and indirect effect estimates across value combinations of $\beta_S$ and $\rho$. Changes in $\beta_S$ had the strongest impact on effect estimates at $\beta_S$ values < 0.042 (figure 2.6).

Across the full parameter space of these model runs, where the direct effect is again constant, the indirect effect of treatment was found to range from -0.43 to 0.88 and the overall effect of treatment ranged from 0.01 to 0.89. Additional model iterations were stratified by $\beta_S$ values and descriptive statistics calculated for each strata, where four strata were defined as low transmission ($\beta_S < 0.014$), moderate transmission ($\beta_S 0.014$ to 0.021), high transmission ($\beta_S 0.0245$ to 0.0315), and extreme transmission ($\beta_S > 0.0315$)
Figure 2.6 Impact of changes in transmission parameter ($\beta_s$) on effect estimates across the full parameter space of $\delta$ and $\rho$. a. indirect effect estimates; b. overall effect estimates. Points are mean values of the effect estimate for 10,000 simulations for each of 30 $\beta_s$ values, error bars are standard deviation.
(table 2.4). All indirect effect estimates were positive at values of $\beta_S \leq 0.021$, noting that as $\beta_S$ approaches zero so do the indirect effects (figure 2.6a). In the range of $\beta_S$ values from 0.0035 to 0.014 the mean indirect effects across the full parameter space of $\delta$ and $\rho$ was 0.516 (s.d. 0.22, min= -0.128 max= 0.882), and mean overall effect was 0.608 (s.d. 0.158, min=0.219 max=0.890). At approximately $\beta_S > 0.028$ the mean indirect effects are observed to become negative for any $\delta \geq 2$.

<table>
<thead>
<tr>
<th>Transmission parameter estimate ($\beta_S$)</th>
<th>Indirect Effect</th>
<th>Overall Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (n=1125)</td>
<td>Mean (s.d.)</td>
<td>Min</td>
</tr>
<tr>
<td>0.505 (0.172)</td>
<td>0.276</td>
<td>0.849</td>
</tr>
<tr>
<td>Moderate (n=1125)</td>
<td>0.253 (0.416)</td>
<td>-0.321</td>
</tr>
<tr>
<td>High (n=1125)</td>
<td>-0.165 (0.006)</td>
<td>-0.379</td>
</tr>
<tr>
<td>Extreme high (n=7500)</td>
<td>-0.315 (0.073)</td>
<td>-0.426</td>
</tr>
<tr>
<td>All values</td>
<td>-0.151 (0.325)</td>
<td>-0.426</td>
</tr>
</tbody>
</table>
Discussion

In simulating the impact of treating chronic subclinical mastitis during lactation using clinically relevant parameter estimates, the overall treatment effect was positive across all parameter values. However, under scenarios where transmission rates have been associated with mastitis outbreaks, in populations of quarters where teat end disinfection was not applied, the overall effect estimates were generally less than 0.20 and approached zero (figure 2.6b). We defined these transmission rate scenarios as high and extreme in this study, and under these scenarios the indirect effect of the intervention was estimated to become negative. Under high transmission scenarios it appears that increasing cure rates from the chronic state increases the size of the recovered susceptible pool, thus contributing to a higher proportion of new infections at equilibrium compared to scenarios with lower cure rates (figure 2.5b).

Previously, Zadoks et al. (2002) reported the potential indirect benefit of increasing the cure rate of subclinical *S. aureus* infections through prompt diagnosis and treatment. In that study the transmission parameters ($\beta_S$) and $\delta$ were 0.007 and 6, and 0.014 and 3.7, for two herds respectively. These parameter values were in the range of values where treatment was predicted to have a positive indirect effect in our current study, and simulation results in our current study were consistent with those of the previous report (compare figure 2.2 in this study to figure 5 in Zadoks et al. (2002)). Further, using herd specific parameter estimates obtained from peer-reviewed publications our current model was able to predict quarter level infection prevalence proportions that are approximately equivalent to the reported true prevalence for those
herds (data not shown). This is not surprising, as the current model structure is similar to
the transmission models that were used to generate the herd specific estimates (Lam et al., 1996; Zadoks, 2002; White et al., 2006).

In economic models Swinkels et al. (Swinkels et al., 2005a; Swinkels et al.,
2005b) recognized the potential economic impact of indirect effects of mastitis treatment. In those studies the authors estimated the indirect effect of treatment as a constant
function across two transmission scenarios. Data from our current study would suggest
the proportion of prevented infections associated with treatment is not constant, but a
complex function of cure rates, transmission rates, and the difference in the force of
infection for naïve susceptible and recovered susceptible quarters. Our results suggest
that there may be a range of transmission rates where the indirect effect of lactation
therapy of subclinical mastitis may be positive and this may be important for advisers to
consider when designing treatment programs. The practical implication of these findings
is that the indirect benefit of treating chronic subclinical mastitis in high transmission
scenarios may be limited. In such situations, producers would be better advised to first
focus on mastitis control practices that reduce the transmission rate, or that remove
chronically infected quarters without contributing to increasing the proportion of quarters
in the recovered susceptible state (e.g. culling or drying of cows or quarters). This is
consistent with previous findings of White et al. (2006) who demonstrated that
interventions had a reduced effect in herds with a high transmission rate, and suggested in
these herds “determinants of transmission should be addressed before (or simultaneously
with) application of other interventions.” In contrast, at moderate to low transmission
scenarios (i.e. approximately $\beta_s \leq 0.021$), the indirect, and overall effect estimates are predominately strongly positive. This suggests that dairy producers that have successfully implemented PMTD programs may benefit from the addition of targeted subclinical mastitis treatment programs to further control mastitis prevalence and transmission. Based on our current findings, herds that may benefit the most from such programs are those with transmission parameter estimates in the range of 0.007 to 0.014.

One issue that arises from these observations is the potential difficulty in estimating herd specific mastitis transmission rates in the absence of extensive longitudinal bacteriologic monitoring. The transmission parameter $\beta$ is a function of the contact rate ($c$) between susceptible and infectious individuals, and the transmission probability ($p$) upon contact (Anderson and May, 1991). These parameters, $c$, $p$, and thus $\beta$, are conditional on our ability to measure exposure to infection, and are frequently difficult to measure directly (Halloran et al., 1997). Estimates of $\beta$ are obtained from longitudinal disease incidence data, and are likely herd specific within a range of values dependent upon milking hygiene practices (e.g. use of PMTD) and dominant species or strains of pathogens causing mastitis, among other potential factors. Additional studies are needed to estimate the transmission parameters of mastitis pathogens and to quantify to what extent recovered susceptible quarters are at increased risk for intramammary infections compared to naïve susceptible quarters. This is relevant to understanding the potential impact of interventions, as Safan et al. (2006) have recently proposed that the ratio (“$r$”) of susceptibility after primary infection and susceptibility after secondary infection is a key parameter in endemic steady state models where backward bifurcation
may occur. Here we have presented an infectious disease model where under some scenarios implementation of an intervention may contribute to higher rates of new infections due to continual filling of the recovered susceptible state.

To the best of our knowledge this is the first study describing a method to quantify the indirect effect of antibiotic treatment interventions for control of mastitis in dairy herds across a range of parameter values with complex interactions. Methods for estimating the direct and indirect effects of interventions have been developed mostly for vaccination trials and are based on ratios of disease risk or infection probabilities in populations receiving the intervention and control populations (Halloran et al., 1997; Haber, 1999; Hayes et al., 2000). Unlike vaccination or hygiene programs which act to reduce the transmission probability ($\beta$), the effect of a targeted antimicrobial treatment program is primarily through reduction in duration of infectiousness. Furthermore, we assumed there to be no extended change in susceptibility of an individual host receiving a course of antibiotic treatment (except during the course of therapy, and for mastitis treatment this can be considered of minor length compared to the total time at risk in the population). However, this assumption ignores the potential that targeted therapy with a narrow spectrum antimicrobial may create an opportunity for infection caused by another species or strain of bacteria that is resistant to the therapeutic. Unlike ‘blanket’ dry cow treatment of all cows at the end of lactations or other mass treatment interventions [e.g. introduction of insecticide treated bed nets, or delivery of antibiotics in defined geographic regions (Hayes et al., 2000; Pourbohloul et al., 2003)], treatment in this model targeted only those individual quarters diagnosed as infected. The indirect effect of the
intervention was assumed to be the reduced risk of infection among susceptible individuals, or as Farrington (2003) described “the efficacy against transmission” and in this study was calculated as the reduction in the proportion of quarters with new infections at equilibrium. Because the determination of indirect and overall effects of an intervention requires comparison of 2 populations, in this study we modeled two populations by duplicating simulations at each of two cure rates to represent a negative controlled field trial where all other factors (i.e. parameters) in the treated and control populations are equivalent. We believe the model has clinical relevance in predicting the indirect and overall impacts of antibiotic treatment interventions during lactation. An advantage to these methods is the ability to explore a large range of scenarios. Future work on evaluating the population level effects of lactation therapy should include completion of clinical field trials. Given our present results, such trials might be initially conducted in herds that, based on herd management practices, are expected to have transmission parameters in the range where indirect effects of treatment may be positive.

Conclusion

Indirect and overall effects have been described as important measures in evaluations of the efficacy of mastitis treatment interventions in dairy cattle populations. This model provides evidence of the potential complex relationship between biologically relevant parameters that may impact indirect effect estimates, and the importance of variation in these parameters, especially the transmission parameter. A key conclusion is that positive population level effects of lactation therapy may be realized for herds that
have successfully implemented practices that reduce the transmission rate of pathogens, while in herds with high pathogen transmission rates, targeted treatment of chronically infected quarters may have little impact on the proportion of infected quarters and no positive effect in reducing new infection rates.

Acknowledgements

This project was supported by USDA Cooperative State Research, Education, and Extension Service, National Research Initiative award # 2005-35204-15651. The project was also supported by USDA-CSREES Vermont Agricultural Experiment Station Animal Health and Disease award # VT-AH01011. The authors would like to acknowledge the support of Dr. John Bramley in background discussions regarding this work.
References


different efficacy and effectiveness aspects of vaccines. Am. J. Epidemiol. 146,
789-803.
issues in cluster-randomized trials of interventions against infectious diseases.
Hillerton, J.E., Kliem, K.E., 2002. Effective treatment of Streptococcus uberis clinical
mastitis to minimize the use of antibiotics. J. Dairy Sci. 85, 1009-1014.
Hogan, J.S., Galton, D.M., Harmon, R.J., Nickerson, S.C., Oliver, S.P., Pankey, J.W.,
1990. Protocols for evaluating efficacy of postmilking teat dips. J. Dairy Sci. 73,
2580-2585.
Lam, T.J., 1996. Dynamics of Bovine Mastitis: A field study of low somatic cell count
estimate efficacy of postmilking teat disinfection in split-udder trials of dairy
Lam, T.J., van Vliet, J.H., Schukken, Y.H., Grommers, F.J., van Velden-Russcher, A.,
 teat disinfection in low somatic cell count herds. II. Dynamics of intramammary
Luby, C.D., Middleton, J.R., 2005. Efficacy of vaccination and antibiotic therapy against
Milne, M.H., Biggs, A.M., Barrett, D.C., Young, F.J., Doherty, S., Innocent, G.T.,
Fitzpatrick, J.L., 2005. Treatment of persistent intramammary infections with
for treatment of experimentally induced Streptococcus uberis intramammary
Oliver, S.P., Gillespie, B.E., Headrick, S.J., Moorehead, H., Lunn, P., Dowlen, H.H.,
extended ceftiofur intramammary therapy for treatment of subclinical mastitis in
antibiotic therapy during lactation and results of antimicrobial susceptibility tests
Pourbohloul, B., Rekart, M.L., Brunham, R.C., 2003. Impact of mass treatment on
syphilis transmission: a mathematical modeling approach. Sex. Transm. Dis. 30,
297-305.


Zadoks, R.N., Allore, H.G., Barkema, H.W., Sampimon, O.C., Wellenberg, G.J., Grohn,


Effects of Lactation Therapy on *Staphylococcus aureus* Mastitis demonstrated through a Field Trial and Molecular Strain Typing

John W. Barlow*, Cameron Nightingale†, Brad Rauch†, Sharinne Sukhnandan†,
A. John Bramley‡, Ruth N. Zadoks§, and Ynte H. Schukken†

*Department of Animal Science, University of Vermont, Burlington 05405
†Quality Milk Production Services, Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca NY 14850
‡Current address: Pickert Dairy, LLC, Bethoud, CO
§Current address: Windham Foundation, Grafton, VT
§Current address: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, UK

For Submission
Abstract

A split herd negative controlled field trial was conducted on 2 commercial dairy herds to evaluate the direct and indirect effects of a diagnosis and treatment program targeting *Staphylococcus aureus* subclinical mastitis. Monthly somatic cell count and bacteriologic culture were used to identify cows with chronic subclinical mastitis. The trial included a 3 month observation period followed by a 10 month intervention period. Pulsed-field gel electrophoresis (PFGE) was used to determine strain specific *S. aureus* infection dynamics in treated and control groups. The direct effect of extended (8 days) intramammary lactation therapy with pirlimycin hydrochloride was demonstrated by an increased proportion of cure and an overall reduction in duration of infection in quarters receiving treatment compared to untreated controls. A reduction in the incidence of clinical *S. aureus* mastitis in the treated group was observed on one farm, while no clinical *S. aureus* mastitis was observed on the second farm. New post-treatment clinical and subclinical infections caused by different PFGE types of *S. aureus*, *Streptococcus* spp., or by coliform species were observed in 50% of quarters that received extended therapy. These infections appeared to occur either during, within the week immediately following, or more than 3 weeks following treatment, and likely influenced the observation that there was no effect of treatment on cow level SCC at 45 days post treatment. The proportion of mastitis associated culls in treated and control groups was not different. The incidence of new *S. aureus* IMI in the treatment and control groups was
not significantly different when data was pooled over farms, PFGE types, and time. Elimination of new infections caused by the dominant strain type on one farm, and reduction in IMI incidence in the first 6 months of the intervention period on the other farm, were consistent with the presence of an indirect effect of therapy. New infections in recovered susceptible quarters, and the emergence of a new PFGE type on one farm influenced incidence during the final 4 months of the intervention period. PFGE of pre- and post treatment isolates, as well as representative isolates taken over the duration of all IMI, provided more precise estimates of new infection, cure, and re-infection rates. This study provides evidence that treatment of subclinical *S. aureus* mastitis during lactation may be beneficial in some herds or for some strain types as a result of both direct and indirect treatment effects. The finding of frequent post-treatment infections is a concern and significantly limited the potential value of extended pirlimycin therapy on these farms. Application of lactation therapy programs may require bacteriologic monitoring to identify potential shifts in the dominant strain types, and infection of recovered susceptible quarters may negate the benefits of extended intramammary treatment during lactation.
Introduction

Mastitis continues to be recognized as one of the most economically important health problems of dairy cattle (Allore and Erb, 1998; Ott and Novak, 2001). Subclinical mastitis, characterized by an elevated milk somatic cell count (SCC), is the dominant form, and frequently goes undetected and untreated by dairy producers for extended periods (Bramley and Dodd, 1984; Hillerton et al., 1995; Oliver et al., 2004). Increased somatic cell count is associated with reduced milk production, increased risk of antibiotic residue violations, reduced fluid milk quality, and reduced cheese yield and quality (Reneau, 1986; van Schaik et al., 2002; Santos et al., 2003). Reduced productivity and lost milk quality payments associated with subclinical mastitis represent more than half of the estimated $1.8 billion in costs attributed to mastitis for U.S. dairy producers annually (Ott and Novak, 2001). The association between the increased somatic cell count due to subclinical mastitis, and reduced productivity and milk quality presents a potential economic opportunity for dairy producers through implementation of control programs targeting subclinical mastitis.

Current mastitis control recommendations are intended to reduce the duration of infections and prevent new infections. Management procedures to accomplish this include the use of long acting antibiotics in all quarters of all cows at the end of lactation (dry-cow therapy), and application of a post-milking teat disinfectant following each milking (Neave et al., 1969; Bramley and Dodd, 1984; Hillerton and Berry, 2005). Treatment of mastitis during lactation has been predominately limited to the treatment of clinical cases, as the economic value of lactation therapy to treat subclinical mastitis is
debated (McDermott et al., 1983; Reneau, 1986; Swinkels et al., 2005a; Swinkels et al., 2005b). A number of reports have described the direct effect of lactation therapy for subclinical mastitis caused by the major gram-positive pathogens \textit{Staphylococcus aureus} and \textit{Streptococcus uberis} [e.g. Deluyker et al., (2005) and Gillespie et al., (2002), and summarized in Barlow et al., (J. Barlow, unpublished data – manuscript submitted, Chapter 2 of this dissertation)]. Delaying treatment of subclinical mastitis until the end of lactation increases the duration of infection. An increased duration of infection reduces the probability of cure (reviewed in Barkema et al., 2006), and may increase the risk of exposure for uninfected quarters as cases of subclinical mastitis may constitute a reservoir of bacteria that are an important source of infection in a herd (Zadoks et al., 2001a; Zadoks et al., 2002a). Hence, curing cases of subclinical mastitis has a direct effect on the individual animal, but may also have an indirect effect on other animals in the same herd, as eliminating infections reduces transmission risk or the force of infection of contagious pathogens (Zadoks et al., 2002a; Barkema et al., 2006; White et al., 2006; J. Barlow unpublished data - manuscript submitted, Chapter 2 of this dissertation)]. Other potential benefits of treating subclinical mastitis may include reduced clinical mastitis by prevention of clinical flare-ups, improved milk quality through SCC reduction, decreased mastitis associated culling, and increased milk production. In a pair of partial budget models Swinkels et al. (2005a; 2005b) recently concluded that lactation therapy of subclinical mastitis may be economically justified in some situations, particularly in high transmission scenarios. However, Barlow et al. showed that there was a curvi-linear effect of the transmission potential on the size of the indirect treatment
effect (J. Barlow, unpublished data – manuscript submitted, Chapter 2 of this dissertation). Using a deterministic disease transmission model, it was suggested that under moderate transmission scenarios the indirect treatment effect was indeed high and important, however, perhaps paradoxically, under very high transmission scenarios the indirect effect of therapy may be close to or less than zero. Previously reported estimates regarding potential indirect effects of lactation therapy are based on observational studies and disease transmission and economic models derived from those studies (Lam et al., 1996; Lam et al., 1997b; Zadoks et al., 2001a; Zadoks et al., 2002a; Swinkels et al., 2005a; Swinkels et al., 2005b). To date no controlled field trials have been conducted to test whether the predicted indirect effects of subclinical mastitis treatment strategies can be demonstrated on commercial dairy farms. Furthermore, there is limited knowledge on the impact of such interventions on the population dynamics of specific bacterial strains. Recent reports have suggested differences among S. aureus strain types in response to therapy (Barkema et al., 2006; Haveri et al., 2007), and in risk of transmission (Smith et al., 1998).

In this research a clinical field trial was designed to evaluate the potential direct and indirect effects of a diagnosis driven treatment program targeting subclinical mastitis. In this report we describe the results of targeted lactation therapy of chronic subclinical S. aureus mastitis, and include pulsed field gel electrophoresis (PFGE) typing to identify strain specific S. aureus infection dynamics. Outcomes evaluated in this study included infection prevalence and incidence, rates of clinical mastitis and mastitis associated culling, and impact on cow level somatic cell count (SCC).
Materials and Methods

Study design

The negative-controlled treatment trial was conducted for a period of 13 months in two commercial dairy herds (Herd 1 was located in New York and Herd 2 in Vermont). Herd size, average milk production, and bulk tank milk somatic cell counts (BTSCC) are shown in table 3.1. Groups of approximately 100 lactating cows were housed in separate pens in free-stall housing on each farm (table 3.1). Cows on each farm were milked 3 times per day in a milking parlor. Criteria for participation included: 1) reliable individual cattle identification based on sequential identification numbers assigned at birth or entry into the lactating herd; 2) housing of lactating dairy cattle in two or more comparable groups (“strings”) of approximately 100 cows in separate pens (“free-stall housing”); 3) enrollment in a Dairy Herd Improvement Association (DHIA) monthly testing program including SCC; 4) an average monthly herd somatic cell count between 250,000 and 500,000 cells/ml; 5) accepted mastitis control practices applied to all cows, including use of pre- and post-milking teat disinfectant solutions, and blanket use of dry-cow therapy; 6) segregated housing for lactating cows receiving antibiotic treatments (i.e. a treated cow pen); 7) ability to keep written records on all cows, including events and dates of calving, entries and exits from lactating cow pen groups, clinical disease, treatment, and culling; and 8) ability and willingness to collect and store milk samples associated with these events. Herd owners were financially compensated.
for participation. The study was conducted with approval of the University of Vermont Institutional Animal Care and Use Committee (IACUC).

The treatment unit was the group of lactating cows with treatment randomly allocated to one of 2 designated pens within herds. In the month preceding the start of the study, cows were systematically assigned to pens based on odd or even identification numbers. Cows that calved during the study were assigned to either the treatment or negative control groups (pens) based on odd-even identification number. Within farms, no differences in mean parity, days in milk, or somatic cell count, were found among treatment and control groups at the start of the study by using this assignment method. Groups within herds were a dynamic population, with entries and exits of individuals into the study population following normal management cycles. Dates of all entries and exits to treatment or control pens were recorded for each cow. Pens of cows within each herd were milked in the following order at each milking session: fresh cow group, study treatment group, study control group, additional groups not enrolled in study. The milking system was washed and sanitized between each milking session. There was no rinsing or disinfection of milking units after milking individual cows during a milking session, and neither farm used a back-flushing system.

The farms applied accepted mastitis control practices to all cows including use of iodine based (0.5 to 1 %) pre- and post-milking teat disinfectant solutions, and blanket use of commercially available dry-cow therapy products (herd 1, cephapirin benzathine; herd 2, cloxacillin benzathine). The use of somatic cell count or bacteriologic culture data to segregate chronically infected cows was not practiced on either farm. The use of
bacteriologic culture to aid clinical mastitis treatment decisions was not practiced on either farm. No diagnosis and treatment of subclinical mastitis was practiced on either farm prior to the start of the study. Cows with clinical mastitis were treated with commercially available intramammary formulations of amoxicillin, cephapirin sodium, or pirlimycin hydrochloride on farm 1 and amoxicillin or pirlimycin hydrochloride on farm 2. Lactating cows in herd 1 were bedded on mattresses with sawdust bedding, while in herd 2 lactating cow stalls were deep-bedded with sand. Stocking density of study pens on both farms exceeded number of stalls by 18 to 20%. In both herds dry cows were loose-housed in a separate covered facility on a chopped straw bedded pack.

Milk sample collection and bacteriologic analysis

Composite milk samples were collected monthly by DHIA technicians and processed through the regional commercial testing laboratories for SCC testing. Individual quarter milk samples were collected for microbiologic analysis from all cows in control and treatment groups at the start of the study, at monthly intervals for the duration of the study, and at the end of the study. These quarter samples were collected within 3 days of DHIA monthly composite sample collection. Quarter milk samples were also collected from all cows within 3 days following parturition (fresh sample), immediately following identification of clinical mastitis (clinical pre-treatment sample), immediately prior to treatment of subclinical mastitis (subclinical pre-treatment sample), at any time when cows were added to or removed from the study pens for greater than 24 hours (entry/exit sample), immediately prior to exit from the herd (cull sample), and at...
approximately 7, 14, 21, and 28 days following cessation of any antibiotic therapy (clinical or subclinical post-treatment samples). Monthly sample collection was conducted by trained field technicians, while farm personnel were trained to collect all additional samples following established aseptic methods (Hogan et al., 1999). Samples collected by farm personnel were immediately stored frozen at -20°C and transported frozen to the microbiology laboratory at two-week intervals. Monthly samples were held on ice immediately following collection and during transport to the laboratory, and were stored frozen at -20°C for 24 – 72 hours. All samples were thawed over-night under refrigeration and aerobic bacteriologic culture and interpretation of results were performed according to established guidelines (Hogan et al., 1999). Samples with >3 morphologically distinct colony types were considered contaminated and eliminated from analysis. For all morphologically distinct colony types in uncontaminated samples, the number of colony forming units (cfu) per 0.01 ml was recorded as a one of four categories: 1 to 4, 5 to 9, 10 to 49, or ≥ 50 cfu (Dingwell et al., 2003).

**Infection status**

This study was intended to model a practical treatment program that might be applied on commercial farms where an elevated SCC is used to trigger bacteriologic culture and culture results are used to trigger a treatment decision. Sequential cow level SCC was evaluated to identify cases of chronic subclinical mastitis which was defined as SCC ≥ 200,000 cells/ml for 2 of the past 3 serial monthly composite milk samples (Schukken et al., 2003). Chronic subclinical mastitis based on composite SCC results was
used as a criterion for enrollment for treatment as described in the next section, and was distinguished from intramammary infection status based on serial bacteriologic culture of quarter milk samples. Bacteriologic culture was used to define intramammary infection status as previously described by Zadoks, et al. (Zadoks et al., 2002a). Briefly, individual quarters were categorized as infected when positive on bacteriologic culture for a single species based on at least one of the following criteria: 1) \( \geq 1000 \text{ cfu/ml} \) from a single sample, 2) \( \geq 500 \text{ cfu/ml} \) from two out of three consecutive samples, 3) \( \geq 100 \text{ cfu/ml} \) from 3 of 3 consecutive samples, or 4) \( \geq 100 \text{ cfu/ml} \) from a clinical sample. Samples with bacteria isolated that did not meet the above criteria were defined as incidental isolation events. An individual quarter was defined as having a subclinical IMI when meeting any one of the first three criteria and having no occurrence of clinical mastitis within the past 14 days. A quarter with a subclinical IMI was defined as spontaneously cured when the quarter was negative for the same pathogen on 2 consecutive samples taken over at least a 28 day interval (Zadoks et al., 2002a). This definition was used to limit the number of errors in defining a cure due to the potential of observing a single false negative culture result on serial samples (Morant et al., 1988; Sears et al., 1990). A subclinical IMI was defined as cured following therapy when culture negative for the pre-treatment species or strain type on 4 of 4 post treatment samples taken at 7 (± 2) day intervals (Gillespie et al., 2002). A clinical mastitis case was defined as an abnormality in appearance or consistency of milk, with or without either local and/or systemic signs associated with infection. A new clinical mastitis case or event was identified at the quarter level after that quarter was observed free of clinical signs for \( \geq 14 \) days or if clinical mastitis
occurred within 14 days of a previous case but was caused by a different pathogen species or a different strain within species. A clinical IMI was classified as a bacteriological cure when culture negative for the pre-treatment species or strain on 4 of 4 post treatment samples taken at 7 (± 2) day intervals. The infection status following either spontaneous cure or therapeutic cure was monitored for the duration of the study or until an animal exited the study population. Data on infection status and cure was recorded for all aerobic bacterial species identified during the trial. A re-infection of a quarter that was previously defined as cured followed the above IMI definitions.

Treatment program

Cows assigned to the treatment group that had chronic subclinical mastitis (i.e. an elevated composite SCC ≥200,000 in 2 of 3 of the recent months) in combination with a positive S. aureus culture in at least one quarter in the current month were eligible for subclinical mastitis lactation therapy. Figure 1 outlines the implementation of the treatment program within the study design. All lactating quarters of eligible cows were treated with a commercially available intramammary formulation of the lincosamide antibiotic pirlimycin hydrochloride (Pirsue™, Pfizer Animal Health, New York) at the labeled daily dosage (50 mg) for an extended (8 days) duration of therapy (extended therapy) (Gillespie et al., 2002; Oliver et al., 2003; Deluyker et al., 2005). Cows in the treatment group identified with a subclinical IMI due to a gram-negative pathogen in the
Figure 3.1 Study design and sample collection procedures for a subclinical mastitis treatment trial

Treated group – months 1 to 3
and
Control group months 1 to 13

- Monthly collection of composite milk samples for SCC analysis
  Cows with chronic subclinical mastitis identified by SCC > 200,000 cells/ml in current month and at least 1 of 2 previous months

- Monthly collection of quarter milk samples for bacteriologic analysis
  Data used retrospectively for determination of intramammary infection status based on sequential samples and using definitions of Zadoks et al. (2002)

Treated group – months 4 to 13

- Monthly collection of composite milk samples for SCC analysis
  Cows with chronic subclinical mastitis identified by SCC > 200,000 cells/ml in current month and at least 1 of 2 previous months

  Within 3 days of SCC testing

  - Monthly collection of quarter milk samples for bacteriologic analysis
    Cows identified with chronic subclinical mastitis based on serial SCC results

  - Examination of monthly bacteriologic culture results
    Cows identified with positive bacteriologic culture for Staphylococcus aureus and chronic subclinical mastitis based on serial SCC tests

    - Enrolled for extended therapy treatment with pimaricin during lactation
    - Collection of quarter milk samples post-treatment

    - Monthly collection of quarter milk samples for bacteriologic analysis
      Data used retrospectively for determination of intramammary infection status based on sequential samples and using definitions of Zadoks et al. (2002)
current month were not eligible for extended therapy due to the limited antimicrobial spectrum of pirlimycin. Cows that had received treatment for a case of clinical mastitis within the previous 14 days were not eligible for extended therapy. No additional covariates that might influence treatment success (e.g. cow lactation number or number of infected quarters, or pathogen duration of infection or antimicrobial susceptibility phenotype) were used to select cows for treatment. Treated cows on both farms were segregated in a ‘sick cow’ pen during treatment and subsequent milk withhold periods. A maximum of approximately 6 cows could be enrolled for extended therapy at any particular time due to the small size and number of available stalls in the treated cow pen on each farm. Cows in the control group diagnosed with subclinical IMI and determined eligible for treatment received no extended therapy, nor an excipient infusion. Cows experiencing clinical mastitis in either group were treated using the established practices on the participating farms and records were maintained on the type and duration of therapy. There was a 3-month ‘pre-intervention’ observation period at the start of the study prior to a 10 month intervention period. All data as described above were collected during both the initial 3 month observation and the subsequent 10 month intervention period.

*Species identification and S. aureus strain typing*

Representative presumptive *Staphylococcus* spp. colonies were identified based on growth characteristics, and were transferred to a blood agar plate for isolation and further identification. All catalase-positive, hemolytic, gram-positive cocci were tested
for coagulase activity by tube agglutination methods (Hogan et al., 1999). Following preliminary species identification and prior to further analysis, coagulase positive staphylococci isolates were transferred to broth culture and stored at -80°C on ceramic beads (CryoBank beads, Copan Diagnostics Inc., Murrieta, CA) in tryptone soy broth with 15% glycerol. Bacterial growth from a quarter milk sample was examined for potential variability in hemolysis or pigmentation patterns of presumptive \textit{S. aureus} colonies, and isolates representing any phenotypic variants were stored separately. All stored coagulase-positive gram-positive cocci were tested by PCR amplification of the thermonuclease gene (\textit{nuc}) using established methods to confirm species identity (Brakstad et al., 1992). Within each quarter, representative \textit{S. aureus} isolates were selected from each IMI for strain typing by PFGE. All isolates from single isolation events were included. In quarters having more than one isolation event over time, isolates for strain typing were selected from early, middle, and late time points. In addition, pre- and post-treatment isolates were selected for strain typing for all cows receiving lactation therapy.

PFGE typing was conducted as described by McDougal et al. (2003), with slight modifications. Isolates were grown overnight on trypticase soy agar plates containing sheep blood and 0.1% esculin. A single colony was inoculated into 5 ml Brain Heart Infusion broth (Becton-Dickinson, Sparks, MD) and incubated overnight at 37°C. Overnight cultures were adjusted to an optical density of 1.3 to 1.4 using a spectrophotometer (SmartSpec Plus, Bio-Rad, Hercules, CA) at 610 nm. Eight hundred µl of the adjusted cell suspension was centrifuged at 13,000 rpm for 5 minutes and the
supernatant was removed. Pellets were re-suspended in 300 µl of Tris-EDTA (TE) buffer (1M Tris-HCL, pH8; 0.5M EDTA, pH8), equilibrated in a 37°C water bath for 10 min and mixed with 10 µl lysostaphin (1mg/mL in 20mM of sodium acetate, pH 4.5, Sigma-Aldrich, St. Louis, MO). The suspension was then mixed with 300 µl of 1.8% SeaKem Gold agarose (Lonza, Rockland, MD) in TE buffer (equilibrated to 55°C) and dispensed into the wells of the large plug molds (Bio-Rad, Hercules, CA). Once solidified, plugs were transferred to tubes containing 3 ml of EC lysis buffer (6mM Tris HCl, 1M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium-lauroyl-sarcosine) and incubated at 37°C overnight. Plugs were washed 5 times in TE buffer on an orbital rocker for 30 min at a time and stored in 4 ml TE Buffer at 4°C. The plug was sliced to 2 x 10mm and the slices were equilibrated in 1X restriction buffer (New England BioLabs, Ipswich, MA) for at least 30min. After removal of the buffer, the restriction enzyme digestion was performed using 1.5 µl of SmaI (20U/µl, New England BioLabs, Ipswich, MA) in 1X buffer at 25°C overnight. Plugs were loaded into wells of a 1% SeaKem gold agarose gel in 0.5X TBE buffer (0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA pH 8.0; Fisher Scientific, Fairlawn, NJ). The wells were sealed with 1% molten agarose. Gels were run for 21 hours at 14°C using a CHEF-Mapper (Bio-Rad, Hercules, CA) at 6.0 V/cm with initial switch time 5 seconds, final switch time 40 seconds, linear ramping and angle 120°. After the electrophoresis was completed, the gel was stained in a 1µg/mL ethidium bromide solution (EMD Chemicals, San Diego, CA) for 30 min. and destained in fresh distilled water for 30 min. Results were documented using the GelDoc 2000 (Bio-Rad, Hercules, CA). PFGE types were identified by visual
examination of gels by two independent observers using established criteria (Tenover et
al., 1995; Zadoks et al., 2002b), with different types identified by > 3 band differences on
restriction digest patterns and indicated by an arbitrary numeric code. Isolates that
differed by 1 to 3 bands were defined as a subtype and indicated by a lowercase letter
following the type number designation.

*Duration of infection*

Duration of infection was calculated based on mid-point estimation method
previously described by Zadoks et al. (Zadoks et al., 2003), where start of the IMI was
defined as the middle of the time interval between a negative culture and the first positive
culture event, and end of the IMI was defined as the middle of the time interval between
the last positive culture and the first negative culture event for a quarter defined as cured.
Quarters that entered the study infected, either at the start of the study or at the start of
lactation, were considered left censored IMI and the start date was the date of first
positive culture at time of entry. Infected quarters that exited the study were considered
right censored and the IMI end date was the date of exit or the end date of the study for
those lactating quarters still infected on the last monthly sample date. Quarter days
infected (IMI prevalence days) in the study pens were calculated based on recorded dates
of IMI start and end. Quarter days susceptible (i.e. days at risk for new IMI among
uninfected quarters) were calculated based on recorded dates of cow entry and exit from
the study pens accounting for the date of changes in quarter infection status during each
interval. Non-lactating quarters in lactating cows were identified with the date of milking
cessation of the individual quarters (i.e. quarter ‘culling’) so that data on infected or susceptible quarter days accounted for any ‘blind’ or culled quarters within lactating cows.

Statistical methods

All statistical analysis was conducted using SAS version 9 (SAS Institute, Inc., Cary, NC, USA). Pearson chi square or Fisher’s exact test were used to test for associations between treatment group and the proportion of quarters becoming re-infected following cure, or the proportion of quarters displaying at least one clinical mastitis event, or the proportion of quarters culled due to mastitis. For the purpose of comparison of proportions and estimating odds ratios for clinical mastitis events only the first *S. aureus* clinical mastitis event was considered, although repeated clinical events within quarters were reported to quantify the total number of clinical mastitis events in treatment and control groups. The hypothesis that treatment affected duration of infection was tested using the Log-rank test to compare the Kaplan-Meier survival function for treated and control *S. aureus* IMI, while controlling for the effect of PFGE type. We also modeled the effect of treatment on duration of infection using a linear regression model including PFGE type as a covariate. Lactation duration for treated and control *S. aureus* IMI was also modeled using Kaplan-Meier survival methods accounting for censored events, using DHIA records of days in milk to define current lactation duration at either exit from the study or the end of the study.
Prevalence of *S. aureus* IMI (number of quarters days infected, $P_T$), and incidence of new *S. aureus* IMI (number of new IMI, $I_N$), were analyzed as outcomes using generalized linear models (PROC GENMOD, SAS) and model checking included examination of deviance and Pearson Chi-square for goodness of fit (Lam et al., 1996; Zadoks et al., 2002a). Evidence of overdispersion was adjusted using Pearson Chi-square estimates divided by the degrees of freedom (Pscale option). Binomial, Poisson, or Negative Binomial error distributions were selected for final models after comparisons for goodness of fit. Backward elimination of variables was used to select the final models with treatment group (*Group*), the effect of interest, forced into all final models and additional independent variables and their 2- and 3-way interactions included when the probability of significance was $<0.10$. Additional variables included in the full models were farm, time period (*Timep*), and PFGE type (*PFGEtype*) where prevalence was the dependent variable, and these same variables plus prevalence ($P_T$) where incidence was the dependent variable. Time period was modeled with 2 categories defining the pre-intervention (observation) period as 0 and the intervention period as 1. Because we observed differences in strain dynamics over time, we also modeled time period with 3 categories, by separating pre-intervention (*earlytp*, months 1-3), early (*midtp*, months 4-8) and late (*latetp*, months 9-13) intervention periods. In the final prevalence model, the impact of treatment group on the prevalence in each interval was modeled assuming a negative binomial distribution, a log link, and an offset of the total number of quarter days,

$$
\varepsilon [\ln(P_T)] = intcpt + Group + Timep + Group * Timep + \ln(N) \quad [3.1],
$$
where \( \varepsilon = \) expected value and \( \ln(N) = \) offset. Comparison of least square means IMI quarter-days prevalence (\( P_T \)) between treatment and control groups and across observation and intervention time periods was conducted separately for each farm using model 3.1 and accounting for multiple comparisons with Bonferroni methods.

The impact of treatment on incidence in each interval was estimated in a Poisson regression model, with a Poisson error distribution, a log link, and an offset of the number of quarter days susceptible for new IMI (\( S \)),

\[
\varepsilon[\ln(I_N)] = \text{Intcpt} + \text{Group} + \text{Timep} + \text{Group} \times \text{Timep} + \ln(S)
\]

[3.2]

Since prevalence was affected by treatment, prevalence was not included as a covariate in model 3.2.

Somatic cell count data was log transformed (LSCC) and the effect of treatment on LSCC was estimated using a generalized linear model (PROC MIXED, SAS) with lactation number category (lactation 1, lactation \( \geq 2 \)), days in milk category (1-90, 91-180, >180), and post-treatment IMI species (none, major gram-positive, coliform) included as covariates.

Transmission parameters (\( \beta_i \)) were estimated from a generalized linear model with number of new IMI events in each monthly interval (\( I_N \)) as the outcome, a log link, assuming a Poisson distribution, and offset \( \ln(S^*I/N) \) (Lam et al., 1996; Zadoks et al., 2002a),

\[
\varepsilon[\ln(I_N)] = \beta_i^* + \ln(S^*I/N)
\]

[3.3]

where \( \beta^* = \ln(\beta) \), \( S = \) quarter days susceptible, \( I = \) quarter days infected, and \( N = \) total quarter days in each interval. Separate transmission parameters (including 95%
confidence intervals) were estimated for the individual farms, as well as between treatment periods, groups, and for PFGE types. The univariate effect of farm, treatment group, time period, and PFGE type on transmission parameter estimates were estimated by inclusion of these variables in model 3.3.

Results

*Intramammary infections eligible for therapy in treated and control groups*

A total of 31,761 quarter milk samples were collected from the two farms, with *S. aureus* isolated from 330 samples. On farm one, there were 14,467 quarter samples taken from 3675 sample events from 385 cows, with 209 (1.4%) samples identified as contaminated leaving 14,258 quarter samples for analysis. Of these, *S. aureus* was isolated from 258 (1.8%) samples. On farm two, there were 17,294 quarter samples taken from 4387 sample events from 580 cows, with 787 (4.6%) samples identified as contaminated leaving 16,507 quarter samples for analysis. Of these, *S. aureus* was isolated from 72 (0.4%) samples.

On farm 1 there were 217 *S. aureus* isolates associated with 48 IMI in 46 quarters of 41 cows, and 37 incidental isolates from 33 quarters of 28 cows that did not meet our definition of IMI. On farm 2 there were 59 *S. aureus* isolates associated with 13 IMI in 12 quarters of 10 cows, and 2 incidental isolates from 2 quarters of 2 cows (table 3.1). In addition, 13 IMI isolates and 2 incidental isolates were obtained from milk samples collected from cows sampled at the start of a lactation where either the IMI cured prior to
entry into a study pen or the cow did not enter a study pen during the course of the trial.

The number of isolates associated with an IMI identified in quarters of cows in the treatment and control pens is shown in table 3.1.

Table 3.1 Herd and study pen group size, production, and somatic cell count descriptive data from monthly Dairy Herd Improvement test records for study herds. Total quarter days for uninfected and infected quarters and number of Staphylococcus aureus isolates collected for the 13 month study period.

<table>
<thead>
<tr>
<th>Herd</th>
<th>pen</th>
<th>monthly average lactating cows</th>
<th>monthly average milk production (lbs)</th>
<th>monthly average SCC (cells/ml x1000)</th>
<th>total quarter days at risk</th>
<th>total quarter days infected</th>
<th>number S. aureus isolates (4 quarters/4 cows)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>treatment</td>
<td>96</td>
<td>98</td>
<td>98</td>
<td>416</td>
<td>132284</td>
<td>1385 (30 / 30)</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>319</td>
<td>72</td>
<td>404</td>
<td>132254</td>
<td>2479</td>
<td>137 (21 / 21)</td>
</tr>
<tr>
<td></td>
<td>whole herd</td>
<td>346</td>
<td>77</td>
<td>252</td>
<td>221</td>
<td>312</td>
<td>221 (50 / 50)</td>
</tr>
<tr>
<td>2</td>
<td>treatment</td>
<td>91</td>
<td>93</td>
<td>292</td>
<td>125241</td>
<td>1345</td>
<td>68 (11 / 11)</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>59</td>
<td>91</td>
<td>296</td>
<td>1115265</td>
<td>14</td>
<td>1 (1 / 1)</td>
</tr>
<tr>
<td></td>
<td>whole herd</td>
<td>346</td>
<td>77</td>
<td>252</td>
<td>68 (21 / 21)</td>
<td>4 (1 / 1)</td>
<td></td>
</tr>
</tbody>
</table>

1. Sum of individual quarter days uninfected at risk for new intramammary infection (IMI) based on pen entry and exit dates.
2. Sum of individual quarter days infected based on midpoint estimates of IMI start and stop dates (Zadoks et al. 2003) and pen entry and exit dates.
3. IMI status based on definitions of Zadoks et al. (2002); incidental isolates are those not meeting IMI criteria; number of isolates obtained from whole herd exceeds sum of treatment and control pens due to inclusion of isolates from cows that either cured prior to entry into study pens or did not enter the study pens.

On farm 1 there were 13 quarters in 11 cows in the treatment group that met the criteria for extended therapy, compared to 16 quarters in 12 cows in the control group (table 3.2). On this farm 9 quarters and 7 cows cured following extended therapy compared to no quarters or cows observed to cure spontaneously in the control group.

On farm 2 there were 9 quarters in 7 cows eligible for extended therapy in the treatment group. Two cows either exited the treatment group (1 cow with 3 infected quarters) or spontaneously cured (1 cow with 1 infected quarter) prior to the start of the intervention period. Four of the 5 quarters that received extended therapy on this farm cured. There
were no cows with *S. aureus* IMI that were identified with chronic subclinical mastitis and a *S. aureus* positive culture in the control group on farm 2 (table 3.2). Data in table 3.2 also summarizes the number of *S. aureus* clinical events and the extent to which exits from the study pens of existing IMI (e.g. right censored events) contributed to overall prevalence in the pens and eligibility for lactation therapy.

### Table 3.2 *Staphylococcus aureus* IMI eligible for extended lactation therapy in treatment and control pens

<table>
<thead>
<tr>
<th>Treatment Pen</th>
<th></th>
<th>Cure following subclinical therapy</th>
<th>Cure following clinical therapy</th>
<th>Right censor reason²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>subclinical treated</td>
<td>clinical at MI start</td>
<td>clinical flare-up</td>
</tr>
<tr>
<td>Farm 1</td>
<td>13</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Farm 2</td>
<td>2</td>
<td>5†</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>18</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Control Pen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>Spontaneous Cure</td>
<td>clinical at MI start</td>
<td>clinical flare-up</td>
</tr>
<tr>
<td>Farm 1</td>
<td>16</td>
<td>nt†</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Farm 2</td>
<td>0</td>
<td>nt†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>nt†</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

1. Bacteriologic cure following treatment of subclinical or clinical IMI is based on negative bacteriologic cultures for the pre-treatment PFGE type on 4 of 4 samples taken at approximately 7, 14, 21, and 28 days post-treatment.

2. Right censored IMI are classified to include all IMI that are not cured prior to either exit from a study pen to another lactating group (exit), dry-off (dry), culling (cull), or at the conclusion of the study (s.end).

3. Spontaneous cure of an IMI is based on 2 sequential negative monthly cultures for the prior PFGE type.

4. nt, not treated, subclinical chronic IMI eligible for therapy in control pens were not treated.

* Two cows with 4 chronic IMI exited the treatment pen prior to the start of treatments in month 4. One cow with 3 infected quarters exited the treatment pen in the third study month due to a management decision (move to low production group), and 1 quarter spontaneously cured during month 2 of the study.
Table 3.3 summarizes the number of clinical and asymptomatic IMI in each pen that did not meet the criteria for extended lactation therapy. On farm 1 in the treatment and control pens these included short duration clinical episodes that cured following clinical therapy (n=3) or spontaneously cured (n=3), or were right censored (n=13). On farm 2 this included 2 chronic IMI in 2 cows in the treatment pen that never demonstrated an elevated SCC, (one of which spontaneously cured and one which was right censored with the end of the study) and 1 IMI in 1 cow that appeared to spontaneously cure, although this cow was identified as a chronic *S. aureus* IMI beginning 4 months later.

Table 3.3 *Staphylococcus aureus* IMI not eligible for extended lactation therapy in treatment and control pens

<table>
<thead>
<tr>
<th>Treatment Pen</th>
<th>Clinical IMI</th>
<th>Asymptomatic IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cure a)</td>
<td>Right censor reason b)</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>dry</td>
</tr>
<tr>
<td>Farm 1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Farm 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control Pen</th>
<th>Clinical IMI</th>
<th>Asymptomatic IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cure a)</td>
<td>Right censor reason b)</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>dry</td>
</tr>
<tr>
<td>Farm 1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Farm 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

1. Bacteriologic cure following treatment of clinical IMI is based on negative bacteriologic culture for the pretreatment PFGE type on 2 or 4 samples taken at approximately 2 months.
2. Right censored IMI are defined to include all IMI that are not cured prior to either exit from a study pen to another lactating group (dry-off, dry-out), or at the conclusion of the study (540 days).
3. Spontaneous cure of IMI is based on 2 sequential negative monthly cultures for the prior PFGE type.
Direct effect of treatment on IMI prevalence

Differences between treatment groups were observed for the total number of infected quarter days on the two farms (table 3.1). The monthly mean number of infected days per 10,000 quarter days in the treatment group on farm 1 was 101 (s.d. 71.6, range 28 to 236), which was significantly less than the mean number of 175.8 (s.d. 100 days, range 71 to 309) infected days in control group (t value 2.55, p=0.018, figure 3.2). On farm 2 there were only 14 infected days observed in a single month in the control group, compared to a monthly mean of 113 (s.d. 98.7 days, range 0 to 251) infected days per 10,000 cow days in the treatment group (figure 3.2). On both farms changes in infection prevalence were observed over time within treatment and control groups (figure 3.2).

On farm 1, the mean prevalence per 10,000 cow days for the pre-intervention time period (months 1 -3) did not differ between groups, however differences in the prevalence were evident in subsequent months in both groups (figures 3.2 and 3.3). The decline in prevalence in the treatment group on farm 1 from month 2 to 3 was attributed to mastitis associated culling of 2 infected quarters and dry-off of 1 infected quarter. Subsequently in this group from months 4 to 9, four of 6 quarters treated with extended therapy were cured, and the constant low prevalence was due to a balance between emergence of new chronic IMI quarters and cure of existing IMI. However in months 10 through 13, prevalence increased as 3 recovered susceptible and 9 other quarters became infected, which was only offset by cure of 5 of 7 quarters treated with extended therapy (figure 3.2 and table 3.4).
Figure 3.2  Prevalence and incidence of *Staphylococcus aureus* intramammary infections (IMI) in treatment and control pens on two farms over 13 monthly intervals. Total prevalence (data points on line) expressed as number of days infected per 10,000 quarter days (right axis). Number new IMI from naïve susceptible quarters (S, □) and number new IMI from recovered susceptible quarters (R, ■) per 10,000 quarter days at risk (left axis). In treatment pens an initial observation period of months 1-3 was followed by the intervention period during months 4-13 when chronic subclinical IMI received extended therapy, while control pens were observed with no intervention applied for 13 months.
Figure 3.3 Least square mean estimates of prevalence of *Staphylococcus aureus* IMI for three sequential time periods for treated and control groups on two farms. Within a treatment group means with different letters differed between time periods on each farm, and between treatment groups means with different numbers differed within the same time period on each farm (p<0.05, Bonferroni adjusted for multiple comparisons).

The observation that during months 10-13 there appeared to be an increased incidence of new IMI in the treatment group on farm 1 influenced our decision to consider these 4 months as a separate time period for comparison to the observation period (months 1-3) and the early intervention period (months 4-9). In the late intervention period (months 10-
new IMIs in the treatment group were approximately equally caused by 4 PFGE (sub)types (1, 1b, 2, and 2b) while in the earlier time periods subtype 2b was absent from both groups (table 3.4). In comparison, in the control group on farm 1, prevalence of PFGE types 1, 1b, and 2 continued to steadily rise during months 4 through 9, where the least squares mean prevalence estimate for this time period (173.0 days) was significantly greater than the same time period for the treatment group (52.7 days, p=0.007, adjusted for multiple comparisons) (figure 3.3). The least square mean prevalence estimate for the control group for months 10 through 13 (306.8 days) was significantly greater than for months 4 through 9 within the same group (p=0.008, adjusted), but was not significantly greater than for months 10 through 13 in the treatment group (214.5 days, p=0.25; figure 3.3).

On farm 2 the change in prevalence over time was evident, with the least square mean prevalence estimate in months 10-13 (11.8 days) being significantly less than months 1 through 3 (178 days, p=0.0005, adjusted) and months 4 through 9 (128 days, p=0.003, adjusted; figure 3.3). This difference was attributed to cure of 4 of 5 quarters, subsequent post-treatment spontaneous cure of the remaining treated quarter, and dry-off of one infected quarter during months 5 through 8. In this group these cures were partially offset by a new IMI in month 9 and entry of an IMI in month 10. Both of these IMI’s were caused by a new PFGE type, and both spontaneously cured by month 12 (figure 3.2 and table 3.4).
Table 3.4 Strain specific dynamics of *Staphylococcus aureus* IMI on two farms during a field trial of lactation therapy. Number of quarters with an IMI in each monthly sample interval are shown for three epidemiological classes for each PFGE type.

<table>
<thead>
<tr>
<th>Farm 1</th>
<th>treated pen</th>
<th>control pen</th>
<th>control pen</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFGE type</td>
<td>IMI class</td>
<td>sample interval (month)</td>
<td>sample interval (month)</td>
</tr>
<tr>
<td>1</td>
<td>entry</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13</td>
<td>Total IMI</td>
</tr>
<tr>
<td>new</td>
<td>1</td>
<td>1 1 1 1 1 2 1 2 2 4 3 3*</td>
<td>1</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>1a</td>
<td>new</td>
<td>1</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>new</td>
<td>1</td>
<td>1 2 2 2</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>new</td>
<td>1</td>
<td>1 1 1 3 3</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>2a</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>2b</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>new</td>
<td>1</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>1</td>
<td>1 1 1 1</td>
<td>1</td>
</tr>
<tr>
<td>5a</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>1</td>
<td>1 1 1 1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>1</td>
<td>1 1 1 1</td>
<td>1</td>
</tr>
<tr>
<td>8a</td>
<td>new</td>
<td>0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>new</td>
<td>1</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td>entry</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>new</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>prev</td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. 'new', new IMI observed to begin in pen group; 'entry', left censored IMI observed prior to entry into pen group; 'prev', prevalence of IMI. *Number of IMI that are observed as a clinical event are indicated by the number of asterisk in either the new class (new IMI first observed as clinical) or prev class (chronic IMI with clinical flair-up).

Because there were so few *S. aureus* infections in the control pen, comparison of treatments on farm 2 was not possible, and we subsequently modeled the effect of treatment on prevalence using only the subset of data for farm 1 and model 1.1 (table 3.5). In univariate analysis time period was a significant positive predictor, while
treatment group was a significant negative predictor of prevalence. In the final linear model the interaction between time period and treatment group was significant, and the effect of treatment within each intervention period was a reduction in the prevalence for the treated group (table 3.5).

Table 3.5 Regression coefficients for the effect of treatment group on *Staphylococcus aureus* IMI prevalence and new IMI incidence in generalized linear regression models using data from farm 1.

<table>
<thead>
<tr>
<th>model variable</th>
<th>estimate</th>
<th>95% CI limits</th>
<th>chi-square</th>
<th>P</th>
<th>DF</th>
<th>dev.²</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMI prevalence = intercept + group + timep + group*timep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-4.6870</td>
<td>-5.1020</td>
<td>-4.2730</td>
<td>491.3</td>
<td>&lt;0.0001</td>
<td>20 27</td>
</tr>
<tr>
<td>group</td>
<td>-0.035</td>
<td>-0.631</td>
<td>0.561</td>
<td>0.01</td>
<td>0.9000</td>
<td></td>
</tr>
<tr>
<td>midtp</td>
<td>0.540</td>
<td>0.047</td>
<td>1.039</td>
<td>4.6</td>
<td>0.0319</td>
<td></td>
</tr>
<tr>
<td>latep</td>
<td>1.060</td>
<td>0.533</td>
<td>1.591</td>
<td>15.46</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>group*midtp</td>
<td>-1.153</td>
<td>-1.869</td>
<td>-0.437</td>
<td>9.95</td>
<td>0.0016</td>
<td></td>
</tr>
<tr>
<td>group*latep</td>
<td>-0.322</td>
<td>-1.079</td>
<td>0.435</td>
<td>0.7</td>
<td>0.4043</td>
<td></td>
</tr>
<tr>
<td>IMI incidence = intercept + midtp + latep + group<em>midtp + group</em>latep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-10.646</td>
<td>-12.532</td>
<td>-8.760</td>
<td>122.4</td>
<td>&lt;0.0001</td>
<td>21 24</td>
</tr>
<tr>
<td>midtp</td>
<td>1.985</td>
<td>0.015</td>
<td>3.955</td>
<td>3.9</td>
<td>0.0482</td>
<td></td>
</tr>
<tr>
<td>latep</td>
<td>1.727</td>
<td>-0.310</td>
<td>3.764</td>
<td>2.76</td>
<td>0.0966</td>
<td></td>
</tr>
<tr>
<td>group*midtp</td>
<td>-1.318</td>
<td>-2.546</td>
<td>-0.092</td>
<td>4.42</td>
<td>0.0355</td>
<td></td>
</tr>
<tr>
<td>group*latep</td>
<td>0.415</td>
<td>-0.580</td>
<td>1.409</td>
<td>0.67</td>
<td>0.4137</td>
<td></td>
</tr>
</tbody>
</table>

1. group = treatment group (reference value is control pen); midtp = months 4 - 9; latep = months 10-13
2. Deviance

**Direct effect of treatment on duration of S. aureus IMI**

The mean duration of chronic IMI calculated from the observed data using the midpoint estimation method was 135 and 86 days for the control and treatment groups respectively. Least square mean estimates of duration from a generalized linear model accounting for the effect of treatment group, *S. aureus* strain, and the interaction between treatment and strain, yielded 165 and 85 days for control and treatment groups respectively, and the effect of treatment was significant (p=0.0072). In a Kaplan-Meier estimate of the survival function of chronic IMI accounting for censored observations, the median duration of infection was estimated to be 256 and 93 days in the control (n=16)
and treatment (n=22) groups respectively, demonstrating a significant reduction in duration of infection in the groups of cows receiving the intervention while controlling for the effects of strain type (log rank test p=0.0025, figure 3.4).

Figure 3.4 Kaplan-Meier survival function of chronic Staphylococcus aureus IMI from 2 commercial dairy herds accounting for right censored observations and including PFGE strain type as a covariate. The median duration of infection for 22 quarters in the treatment groups (dashed line) estimated to be 93 days was less than the 256 day median duration of infection estimate for 16 quarters in the control groups (solid line) demonstrating a reduction (log rank test p=0.0025) in duration of infection in the groups of cows receiving the intervention while controlling for effects of strain type.
Effect of treatment on rates of S. aureus clinical mastitis and culling due to mastitis

Differences were observed between the treatment and control pens in the incidence of clinical mastitis due to S. aureus, the incidence of mastitis associated culling among S. aureus infected quarters, and number of chronic S. aureus IMI at the end of the study (table 3.2). No clinical S. aureus mastitis was observed on farm 2. On farm 1, 2 of 13 cows (2 of 13 quarters) with chronic S. aureus mastitis in the treatment group had at least one clinical mastitis event, while 7 of 12 cows (10 of 16 quarters) developed S. aureus clinical mastitis in the control group. The odds of a cow with chronic S. aureus mastitis displaying at least one clinical mastitis episode among cows in the control group was 7 times that of cows in the treated group (p=0.025, Fischer exact test, exact confidence limits 0.09<OR< 93) while the odds of clinical mastitis among quarters in the control group was 10 times that of the treatment group (p=0.01, Fischer exact test, exact confidence limits 1.2<OR< 104). On farm 1 there were 3 quarters in 2 cows that displayed > 1 clinical episode for a total of 11 clinical events in this group.

There were fewer total right censored S. aureus IMI in the treatment compared to the control group, including fewer quarters with chronic mastitis that entered the dry period infected or that were still infected at the end of the study (table 3.2). There was a numerical increase in the proportion of mastitis associated culling events from the treatment pen compared to the control pens, but this difference was not statistically significant (Fisher’s exact test, p=0.22). Three cows with 3 S. aureus IMI were culled from the control group, 2 of these quarters with the primary reason for culling being mastitis. Four cows with 6 S. aureus IMI were culled from the treatment group, 5 of these
quarters with the primary reason for culling being mastitis. Among the mastitis culls in
the treatment group, 2 culling events were in recovered quarters that had previously
received extended lactation therapy, with one of these culling events being associated
with a gram-negative infection acquired after \textit{S. aureus} cure.

In a Kaplan-Meier estimate of the survival function, the mean lactation length
among the quarters with chronic \textit{S. aureus} IMI receiving extended therapy was 426
(median 436, se 24) days, which did not differ from the mean lactation length for quarters
with chronic IMI that did not receive extended lactation therapy (mean 420, median 456,
se 27 days), suggesting that treatment was not associated with an increase or a decrease in
lactation duration (log rank test \(p=0.29\)).

\textit{Indirect effects of treatment on \textit{S. aureus} IMI incidence}

There were a total of 42 new IMI per 501,774 quarter days at risk, with 36 new
IMI observed on farm 1 and 6 new IMI on farm 2 (table 3.4). A strongly favorable effect
of treatment was observed in the early intervention period (\textit{midtp}, months 4-9; table 3.5).
In the late intervention period (\textit{latetp}, months 10-13), the incidence rate for the treatment
group was higher compared to the control group but the effect of treatment was not
statistically significant (table 3.5).

\textit{Strain typing}

Eight PFGE types differentiated by \(>3\) band differences were recognized to cause
\textit{S. aureus} IMIs, and 1 to 2 additional subtypes were identified by 1 to 3 band differences
within 5 of these PFGE types (table 3.4). On farm 1, two dominate PFGE types caused IMIs. PFGE type 1 and its subtypes were associated with 25 (52%) IMI in 25 quarters of 24 cows, and PFGE type 2 and its subtypes were associated with 17 (35%) IMI in 17 quarters of 15 cows. On farm 2 a single dominate pulso-type (PFGE type 3) was associated with 8 (62%) IMI in 8 quarters of 6 cows in the treatment group. Among cows with multiple infected quarters, 5 cows had 2 quarters infected with either different PFGE types (n=3) or subtypes (n=2), compared to 1 cow which had 2 quarters infected with the same strain type, and 3 cows which had 3 quarters infected with the same strain type. Chronic infection due to a single strain type or subtype over time was common, with a change in a major strain type identified for 1 chronic IMI (2%), and 5 changes in PFGE sub-types identified in 4 chronic IMI. For the chronic IMI where a major strain type change was observed over time within a quarter, this event was associated with a shift in strain type following lactation therapy for subclinical mastitis. Among the differences in subtypes observed over time within a quarter, 3 of these events were associated with a shift in strain type following lactation therapy for clinical mastitis, and in the other 2 cases, there was no recorded treatment prior to the change in sub-type in the quarter.

Influence of strain typing on determination of bacteriologic cure

One quarter of one cow was observed to be culture negative for 3 weekly post treatment samples, and was subsequently S. aureus positive on 29 days post treatment, and PFGE typing of pretreatment and post treatment isolates demonstrated a type change.
This event was defined as treatment cure followed by re-infection in a recovered susceptible quarter.

There were two IMI (one in each farm) defined as non-cure based on one or more positive cultures with the same PFGE type in the 4 weekly samples following extended therapy that were subsequently found to be culture negative for *S. aureus* in later months. We defined these events as post-treatment spontaneous cures. On farm 1 this was in a quarter that was positive for the same PFGE type on 7, 14, and 21 days post-treatment, but became *S. aureus* negative on day 28 and remained negative for 5 monthly samples over 130 days. On farm 2 this was a quarter that was positive for the same PFGE type on day 7 post-treatment but was *S. aureus* negative on the remaining 3 weekly post-treatment samples and remained negative on 6 serial samples taken over 174 days.

**New IMI in recovered susceptible quarters**

In addition to the new *S. aureus* IMI identified by a PFGE type change following extended therapy described in the previous section, 2 quarters in 2 cows receiving extended therapy targeting a different bacterial species were also observed to become infected with *S. aureus* following extended therapy cure. Thus, we observed 4 new *S. aureus* IMI in recovered susceptible quarters following extended lactation therapy (figure 3.1). Similarly, among the 18 chronic *S. aureus* IMI receiving extended lactation therapy, we observed 8 post-treatment new clinical or subclinical IMI with a different species, including 3 acute clinical IMI due to *E. coli*, 2 acute clinical and 1 chronic subclinical IMI due to *Klebsiella* spp., 1 acute clinical *Streptococcus dysgalactiae* IMI,
and 1 acute clinical event with no bacterial growth detected. Therefore, among 18 quarters in 16 cows with chronic *S. aureus* mastitis that received extended therapy during lactation, 9 (50%) quarters and 8 (50%) cows became re-infected with a major mastitis pathogen. These post treatment IMI occurred either during the 8 days of therapy (n=1) between 3- 5 days after the last treatment 9 (n=4), or at least 16 days after the last treatment (n=4, range= 16 to 84 days). In 7 of these cases the *S. aureus* IMI targeted for therapy was observed to cure. In 2 quarters the *S. aureus* was cultured from the quarter following treatment up until the development of clinical mastitis caused by *E. coli* (at 30 days post-treatment on farm 1) or *Klebsiella* (at 16 days post treatment on farm 2). In comparison, among the 16 chronically infected quarters in the control group only 1 new clinical *E. coli* IMI was observed to emerge during the course of a chronic *S. aureus* infection. The odds of developing acute clinical or chronic subclinical coliform mastitis among the 18 quarters with chronic *S. aureus* mastitis that received extended therapy during lactation were 7.5 times that of the 16 cows with chronic *S. aureus* mastitis that did not receive extended therapy (p=0.05, Fischer exact test, 95% exact confidence limits 0.7<OR<367).

*Effect of extended therapy treatment on somatic cell count*

Pre- and 45 day post-treatment SCC data was available for 13 treated and 10 control cows. The mean LSCC at approximately 45 days post treatment (post sample 2) for the four treated cows that cured *S. aureus* IMIs and were culture negative for an IMI by any major pathogen in the 45 days post treatment was 170,000 (SCC estimate back-
transformed from mean LSCC) cells/ml which was significantly less than the mean pre-
treatment 3 month average SCC of 557,000 cells/ml for these cows (paired t-test for
difference of means, \( p=0.03 \)). All 10 cows in the control group were still \( S. \) aureus
positive at 45 days following the first month of eligibility for treatment and had a mean
45-day post treatment SCC of 746,000 cells/ml which was not different from the mean
pre-treatment 3 month average SCC of 784,000 cells/ml. The mean 45-day post-treatment
SCC of 876,000 cells/ml for cows with a post treatment IMI (either caused by a major
gram-positive pathogen or a coliform species) did not differ significantly from their
pretreatment SCC of 908,000 cells/ml. In a generalized linear model only post-treatment
pathogen category was a significant predictor of the 45-day post-treatment SCC. The
least squares mean LSCC of cows with post-treatment major positive IMI or coliform
IMI was significantly greater than the post-treatment SCC of cows with no post-treatment
IMI and the effect of a post-treatment IMI was to increase the 45 day post-treatment SCC
by approximately 800,000 cells/ml based on the difference of back-converted LSCC
estimates (figure 3.5 and table 3.6).
Figure 3.5 Effect of treatment on somatic cell count (SCC). Least squares mean estimates of the natural logarithm of SCC (LSCC) for mean 3 month pre-treatment and 45-day post-treatment cow level SCC stratified pre-treatment by treatment group, and post-treatment by post-treatment infection status [Pre-treatment IMI caused by *Staphylococcus aureus*; None = bacteriologic culture negative; Major Gram + = intramammary infection (IMI) caused by *Staphylococcus aureus* or a *Streptococcus* spp.; Coliform = IMI caused by *Escherichia coli* or *Klebsiella* spp.]

Table 3.6 Regression coefficients for the effect of treatment group on 45 day post treatment SCC. Model, LNPOST2 = TREATMENT + POST_IMI_PATH

| Variable   | Estimate | Standard Error | DF  | t Value | P>|t| |
|------------|----------|----------------|-----|---------|-----|
| Intercept  | 4.79     | 0.72           | 19  | 6.68    | < 0.001 |
| Treatment  | 0.35     | 0.55           | 19  | 0.63    | 0.535  |
| Control    | 0        |                |     |         |       |
| Major Gram + | 1.82   | 0.66           | 19  | 2.78    | 0.012  |
| Coliform   | 1.94     | 0.52           | 19  | 3.13    | 0.006  |
| None       | 0        |                |     |         |       |
S. aureus transmission parameter estimates

Table 3.7 lists the transmission parameter ($\beta$) estimates for S. aureus from the full data set and for each individual farm. Covariates that were tested in this model included time period, treatment group, farm, and strain type but only strain type was statistically significant, with type 1 having a significantly increased log-odds effect estimate compared to the baseline reference type 3 in model 1.3. Table 6 also lists the transmission parameter estimates for each of the three major PFGE types. The transmission parameter for PFGE type 1, which was isolated almost exclusively from farm 1, was significantly different from the transmission parameter for PFGE type 3, which was exclusively isolated from farm 2, showing that transmission parameters may be specific to herds or bacterial strains rather than to bacterial species.

Table 3.7 Transmission coefficient ($\beta$) estimates for Staphylococcus aureus transmission on 2 dairy farms

<table>
<thead>
<tr>
<th>Data set</th>
<th>Estimate</th>
<th>95% CI limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>full</td>
<td>0.00710</td>
<td>0.00495 0.01019</td>
</tr>
<tr>
<td>farm 1</td>
<td>0.00804</td>
<td>0.00557 0.01161</td>
</tr>
<tr>
<td>farm 2</td>
<td>0.00448</td>
<td>0.00147 0.01369</td>
</tr>
<tr>
<td>PFGE type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.01038</td>
<td>0.00718 0.01499</td>
</tr>
<tr>
<td>2</td>
<td>0.00589</td>
<td>0.00271 0.01280</td>
</tr>
<tr>
<td>3</td>
<td>0.00330</td>
<td>0.00166 0.00552</td>
</tr>
</tbody>
</table>
Discussion

This study demonstrated a positive direct effect of extended lactation therapy for chronic subclinical mastitis caused by *S. aureus*. We observed a significant increase in proportion of quarters with bacteriologic cure among chronically infected subclinical IMI receiving 8-day pirlimycin therapy during lactation compared to the proportion of spontaneous cure in untreated quarters. The criteria for defining cure in our study were comparable to those of Gillespie et al. (2002) who observed 83% cure with 8-day pirlimycin therapy of *S. aureus* mastitis, comparable to 72% cure in our data. Compared to our results, lower proportions of cure have been reported for other pirlimycin treatment regimens targeting subclinical *S. aureus* mastitis [e.g. ranging down to 13% for a 2-day duration of treatment (Gillespie et al., 2002)]. Sol et al. (1997) and Deluyker et al. (2005) have reported on host factors associated with cure after treatment of subclinical *S. aureus* mastitis (reviewed in Barkema et al., 2006). Deluyker et al. (2005) reported cure for *S. aureus* following an 8 day pirlimycin regimen to vary between 10 and 88% depending on host and laboratory covariates, although these authors did not report an overall cure proportion by bacterial species. Previous studies have demonstrated *S. aureus* strain diversity between farms (Kapur et al., 1995; Zadoks et al., 2000; Rabello et al., 2007), and the large number of farms in the Deluyker study could have resulted in an increase in the diversity of *S. aureus* strains. This greater diversity may impact the overall cure rate observed; assuming probability of cure is associated with strain type (Haveri et al., 2007). The potential effect of herd (as a possible proxy for *S. aureus* strain type) was not
evaluated by Deluyker et al. (2005). In our study 5 strain types were treated with extended pirlimycin therapy, and no isolates demonstrated lincosamide or macrolide resistance as measured by disc diffusion or microtiter plate broth dilution methods. Of the 18 quarters treated, cure was observed for 5 of 8, 3 of 4, 4 of 4, 1 of 1, and 0 of 1 quarters for PFGE types 1, 2, 3, 5, and 10 respectively. Interestingly, only PFGE types 3 and 10 demonstrated either phenotypic or genetic evidence of penicillin resistance [based on disc diffusion, microtiter plate broth dilution, nitrocefin testing, and PCR-based detection of genes for 100 % (n = 19) of pre-treatment isolates examined from the 5 cows]. For PFGE type 3 penicillin resistance did not appear to affect the probability of cure following pirlimycin treatment, although previous studies have suggested that beta-lactamase resistance is associated with a decreased probability of cure even when non-beta-lactam antimicrobials are used for treatment (reviewed in Barkema et al., 2006).

The relatively small number of IMI caused by *S. aureus* on the two farms in our study and the high proportion of cure among treated quarters limited our ability to identify significant host or strain effects associated with cure (data not shown).

On farm 1, treatment was strongly associated with a reduced incidence of *S. aureus* in the early intervention time period (incidence rate of the treatment group estimated to be approximately 27% of that of the control group in months 4 to 9). In this time period treatment was also associated with a reduction in *S. aureus* prevalence. In the later intervention time period, the incidence rate in the treatment group was estimated to be approximately 1.5 (95% CI 0.25 to 4.1) times that of the control group, which was not
significantly different. In this late time period prevalence in the treatment group was also not significantly different from the control.

On farm 1 in the later intervention period (months 10-13) there were 2 times as many new IMI in the treatment group compared to the control group. In the treatment group at least 3 and up to 7 out of 10 new IMI emerged during months 10 and 11 in the absence of any existing IMI of the same PFGE-type in the current or previous months. In comparison only 1 of 2 new IMI emerged with no prior existing prevalence in the control group during the same months. Further, 4 of 7 new IMI in month 10 in herd 1 were caused by a previously unobserved PFGE sub-type. Similarly, in herd 2 following elimination of the dominate PFGE-type by month 8, a new ‘sporadic’ IMI due to a previously unobserved PFGE type emerged in the treatment pen, and a single new IMI was observed in the control pen with no prior existing infections of this type. These data suggests that new *S. aureus* IMI likely result from a combination of contagious transmission from existing intramammary infections and sporadic infections of extra-mammary origin (Zadoks et al., 2002b).

Although we can not completely eliminate the possibility of contagious transmission between cows in separate pens, the design of this study included control of pen milking order. Milking systems were washed and sanitized between each milking session, and only the pen of fresh cows was milked prior to the treatment group and infection dynamics were monitored over the two week period that cows resided in the fresh pen. The possibility of spread of pathogens from the treatment group to cows in the
control group via contamination of the milking system can not be eliminated; however the reverse scenario was unlikely due to the pen milking order.

The origin of sporadic cases may include the cow’s skin, the milking or housing environment, or milkers’ hands (Zadoks et al., 2002b; Smith et al., 2005b). Because we only examined quarter milk samples, the epidemiology of any transmission from extra-mammary sources could not be evaluated in this study. While strain typing can be sufficient to demonstrate that new infections were not the result of contagious transmission (Zadoks and Schukken, 2006), if extra-mammary sources of S. aureus are to be identified as a source of sporadic infections, future studies should include environmental surveillance. This may be especially valuable in low prevalence herds where sporadic infections may constitute a greater proportion of new IMI relative to those resulting from contagious transmission.

An important observation from this study is the report of new S. aureus infections caused by a different strain type in previously cured quarters. Unique to our study and that of Luby and Middleton (2005), strain typing of pre- and post-treatment S. aureus isolates provided improved estimates of cure and re-infection proportions. In the absence of strain typing we would have under-estimated both the cure proportion observed and the re-infection rate. Our ability to discriminate among strains led to the observation that a proportion of presumed non-cures based on bacteriologic culture alone (1 of 6 quarters found S. aureus positive post-treatment in this study), may actually be cure followed by a new post-cure infection with a different strain type. Based on routine bacteriology, the observed cure proportion in this study would have been 78% compared to 72% based on
strain typing. Barkema et al. (Barkema et al., 2006) and Zadoks and Schukken (Zadoks and Schukken, 2006) have previously suggested that reports of cure rates in the absence of strain specific data may under-estimate true cure rates due to re-infection of cured quarters by contagious transmission from infected quarters in either the same cow or other cows in a herd. Luby and Middleton (2005) had previously shown this for 1 of 16 quarters enrolled in a treatment trial. Our results provide further support of this concept, although we can not further distinguish between cure followed by re-infection and non-cure for the 5 quarters infected with the same PFGE-type post-treatment. In addition, we can not eliminate the possibility of genetic changes resulting in a change in PFGE type following treatment in a non-cure IMI (Goerke et al., 2004), which may explain the subtype changes observed in following both clinical and extended subclinical therapy during our study, although not all subtype changes occurred following antimicrobial therapy. Also, the possibility of carriage of more than one strain and isolation of different strains pre-treatment and post-treatment or across a chronic infection can not be eliminated (Smith et al., 2005a).

The intervention did have a positive effect in reducing the rates of S. aureus clinical mastitis which we hypothesize is related to the effect of treatment reducing duration of infection of chronically infected quarters. This is supported by the observation that the difference in S. aureus clinical mastitis rates between the treatment and control groups on farm 1 was primarily a result of a higher number of clinical flare-ups among existing chronic infections in the control group. Our finding of an effect of lactation therapy for subclinical mastitis being associated with a reduction in the number
of clinical mastitis cases is in agreement with St. Rose et al (St Rose et al., 2003), and may reduce the use of antibiotic treatments for clinical mastitis, offsetting the costs of treating subclinical mastitis (Swinkels et al., 2005a). This is currently controversial, as other authors have recently reported no difference in mastitis treatment rates following treatment of subclinical mastitis compared to an untreated control group (Sandgren et al., 2008).

These positive findings of an effect of treatment on \textit{S. aureus} subclinical and clinical mastitis incidence are countered by the frequent occurrence of new IMI in cows receiving extended lactation therapy which were dominated by clinical coliform mastitis cases within a few days to more than 2 months following cure of the initial \textit{S. aureus} infection. We also observed a numerically higher, but non-significant increase in the number of mastitis associated culling events for the treatment group compared to the control group. Sandgren et al. (2008) also reported an increased rate of udder-health associated culling in cows receiving intramammary therapy for subclinical mastitis. Gillespie et al. (2002) and Middleton and Luby (2008) reported new gram-negative infections or clinical mastitis leading to early dry-off or death of cows following lactation therapy with pirlimycin. These authors suggested this was possibly due to a number of factors including introduction of pathogens during intramammary therapy, use of narrow-spectrum antimicrobials, poor environmental hygiene, and seasonal effects of increased heat and humidity. Introduction of pathogens during therapy was a possibility in a number of cases in our study. Five (56\%) treated quarters developed a post-treatment IMI (4 coliform and 1 no growth clinical cases) within 5 days after the last extended
therapy infusion, and one of these quarters developed subclinical *E. coli* mastitis on the sixth day of treatment based on daily culture of cows during the 8 day treatment regimen. These are presumed to be 5 iatrogenic cases of mastitis, likely associated with a combination of poor hygiene practices during treatment and/or disruption or trauma of the teat canal leading to increased susceptibility, plus the limited antimicrobial spectrum of pirlimycin. One additional quarter developed clinical *S. dysgalactiae* mastitis on day 16 following treatment. Three (33%) quarters that cured of *S. aureus* following extended therapy were observed to become infected by a different *S. aureus* PFGE type (n=1, 30 days post treatment) or a coliform (n=2, 33 and 84 days post treatment) after at least 3 negative post-treatment samples. This may represent a subset of cows where factors in addition to any potential iatrogenic effects are responsible for re-infection. For example, the role of host susceptibility (Lacy-Hulbert and Hillerton, 1995) may also contribute to the risk of post-treatment re-infections in agreement with observations of Zadoks et al. (2002a) who described an increased risk of infection among recovered susceptible quarters.

Treatment clearly eliminated infections and reduced prevalence of *S. aureus* in both herds, however the association between prevalence and incidence could only be compared between treatment and control groups in herd 1. For this herd, we suggest that the association between treatment and incidence was through the intermediate effect of prevalence. This is consistent with a causal model for contagious disease transmission, where the effect of treatment is to reduce duration of infection and thus overall prevalence, which in turn results in a reduced force of infection for the susceptible
individuals. The effect of treatment during the early intervention period (where contagious transmission appeared to dominate) suggests that lactation therapy may provide additional transmission control by lowering the force of infection. The observed ‘outbreak’ in the treatment group on farm 1 during the late intervention period associated with new infections caused by both an existing strain and emergence of a new strain is worth further investigation. This could either be a result of the impact of the treatment program selecting for the emergence of new contagious strain type(s), or may be unrelated to treatment and/or contagious transmission and simply be the result of the unrecognized introduction of an infected animal, or infections acquired from a common environmental source. The small number of infections observed would be consistent with any of these possibilities. Three (23%) of the new IMI that emerged in this late intervention period were in recovered susceptible quarters, indicating that culling or segregation should be considered as an additional control measure for infected quarters that become re-infected or that do not respond to therapy. A comparison of the two herds in this study demonstrates the herd specific nature of *S. aureus* mastitis and possible differences among strains in transmission and response to interventions. Modeling an effect of treatment on IMI prevalence in herd 2 was not reasonable due to lack of IMI caused by PFGE type 3 in the control group for comparison. Inferences regarding infection dynamics in the absence of treatment were restricted to only the first 3 months of the study for this herd and PFGE type.

In a disease transmission model, Barlow et al. previously predicted that the indirect effect of therapy would approach zero under scenarios of very low transmission
parameter estimates (J. Barlow, unpublished data – manuscript submitted, Chapter 2 of this dissertation). From a field trial conducted in the Netherlands, Lam et al. (1996) observed 13 new *S. aureus* IMI per 71,139 quarters days in quarters receiving post-milking teat disinfection and obtained a point estimate of 0.0032 for the transmission parameter $\beta$. In our current study we observed 36 new IMI per 260,238 quarter days at risk in herd 1, and using the same model predicted the ‘pooled’ transmission parameter $\beta = 0.008$, with a 95% confidence interval on this estimate that falls within the range of values where we have previously predicted the indirect effect of treatment to be positive (J. Barlow, unpublished data – manuscript submitted, Chapter 2 of this dissertation). As expected, during the early intervention period we did observe a positive indirect effect of treatment. The size of the indirect effect was in line with our model expectations. Strain typing of isolates allowed estimation of strain specific transmission parameters, although in this study, as in a previous study of three herds (Zadoks et al., 2002a), it was difficult to separate strain effects from herd effects on transmission parameters because strains were largely herd-specific. Recognizing this limitation, potential differences in transmission among strains were identified, with PFGE type 3 appearing to have a lower transmission potential compared to PFGE type 1. Transmission parameter estimates for PFGE types 1 and 2 were ‘pooled’ estimates including the respective sub-types. Differences in transmission have been described for two *S. aureus* strains that occurred during the same time period within a single herd (Smith et al., 1998). Other putative strain associated factors, such as persistency of infection (Haveri et al., 2005; Haveri et al., 2007), may impact the force of infection within a herd (Dodd and Neave, 1970).
Additional research is required to quantify differences in transmission probabilities among *S. aureus* strains associated with bovine mastitis, and the potential relevance of these differences to mastitis control practices.

The decision to implement a subclinical mastitis treatment program during lactation is primarily economic although welfare concerns also exist (Hillerton and Berry, 2003). Swinkels et al. (2005a) have described a partial budget model for estimation of economic benefits of treating subclinical mastitis caused by *S. aureus*. Their model was sensitive to estimates of transmission probability and probability of culling. In addition, the probability of clinical mastitis was a factor contributing to the economic benefits of lactation therapy. Results of our study provide additional information on the use of lactation therapy for control of subclinical mastitis in dairy herds. Key among our findings was the value of strain specific measures of infection and cure rates, and the observation that strain specific infection dynamics influence interpretation of direct and indirect treatment effect estimates. In addition, we demonstrated a reduced rate of clinical mastitis in the treated group on one farm, as well as elimination of IMI caused by a dominant strain in a second herd. These benefits were offset by increased rates of clinical and subclinical mastitis in cured quarters following extended pirlimycin therapy, which appeared to influence the finding that there was no effect of treatment on post-treatment SCC. Alternative subclinical mastitis treatment regimens such as systemic therapy might be considered as a method to prevent potential iatrogenic mastitis associated with intramammary infusion, although it appears this would not eliminate all post treatment
re-infections (Sandgren et al., 2008). The balance of these potential effects needs further consideration in economic models and additional field studies.

Conclusion

This research demonstrated a direct effect of lactation therapy in the overall reduction of duration of *S. aureus* infection on two commercial dairy herds. A significant indirect effect of treatment was also observed, although differences between herds and over time within herds were present. These differences demonstrate the potential for strain and herd specific responses to lactation therapy programs. Our data demonstrate potential benefits of subclinical mastitis treatment during lactation as a component of mastitis control programs on dairy farms, although the high rate of infection of recovered susceptible quarters and the occurrence of post-treatment coliform infections observed in these herds are two issues that would negate the potential benefits of extended pirlimycin therapy of subclinical *S. aureus* mastitis.

Acknowledgements

The authors acknowledge the contribution of participating farm owners and farm staff. The support of our field and laboratory staff is also greatly appreciated. This project was supported in part by funding from Pfizer Animal Health, USDA Cooperative State Research, Education, and Extension Service (CSREES), National Research Initiative
award # 2005-35204-15651, USDA-CSREES Vermont Agricultural Experiment Station
Animal Health and Disease award # VT-AH01011, and the Multi-State Mastitis Research Project USDA CSREES NE-1028 (formerly NE-1009).

References

Staphylococcus aureus from persistent and nonpersistent intramammary infections with different clinical characteristics. J. Appl. Microbiol. 103, 993-1000.


the dairy herd by hygiene and management. J. Dairy Sci. 52, 696-707.
Effect of penethamate hydriodide treatment on bacteriological cure, somatic cell count and milk production of cows and quarters with chronic subclinical *Streptococcus uberis* or *Streptococcus dysgalactiae* infection. J. Dairy Res. 70, 387-394.


Effects of lactation therapy on subclinical

*Streptococcus uberis* and *Streptococcus dysgalactiae* mastitis

John W. Barlow\*\*, Cameron Nightingale\†\†, Brad Rauch\†, Natasha Belomestnykh\†, A. John Bramley\*\*\, Ynte H. Schukken\†, and Ruth N. Zadoks\†\†\†

\*Department of Animal Science, University of Vermont, Burlington 05405

\†Quality Milk Production Services, Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca NY 14850

\†\†Current address: Pickert Dairy, LLC, Bethoud, CO

\*\*Current address: Windham Foundation, Grafton, VT

\†\†\†Current address: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, UK

For submission
Abstract

A split herd negative controlled field trial was conducted on 2 commercial dairy herds to evaluate the direct and indirect effects of a diagnosis and treatment program targeting subclinical mastitis caused by streptococcal species. Treatment of subclinical mastitis by extended (8 days) intramammary lactation therapy with pirlimycin hydrochloride was randomly allocated to a group of approximately 100 cows on each farm, and cows in the treatment group were determined eligible for treatment based on serial monthly somatic cell count (SCC) testing and individual quarter milk bacteriologic analysis. Extended therapy eliminated a greater proportion (71%) of chronic subclinical intramammary infections (IMI) caused by Streptococcus uberis and Streptococcus dysgalactiae compared to either spontaneous cure (50%) of chronic subclinical IMI or cure of clinical episodes (50%). The direct effect of treatment at the cow level appeared to be limited, as there was a high proportion of spontaneous cure of chronic subclinical mastitis among cows that did not receive extended therapy in the negative control group and the overall duration of S. uberis and S. dysgalactiae IMI was not different between treatment and control groups. Treatment affected the prevalence of S. uberis IMI in one herd. A significant reduction in S. uberis new IMI was associated with the time of reduced S. uberis prevalence in this herd, suggesting a potential herd level or indirect effect of treatment. In one herd there were a slightly greater number of infections due to S. dysgalactiae compared to S. uberis; while in the second, S. uberis was the dominant cause of chronic subclinical and clinical streptococcus mastitis. Random amplified
polymorphic DNA (RAPD) testing demonstrated strain diversity among *S. uberis* isolates causing mastitis, consistent with environmental sources of exposure for this pathogen in both herds. Differences in the extent of *S. uberis* strain diversity were observed between the 2 herds, with a single strain type associated with 52% of IMI in one herd, and 26% in the second herd. Species identification and strain typing improved estimates of cure proportions by distinguishing new streptococcus species infections from non-cure following treatment. Five (24%) of the treated quarters developed subclinical or clinical infections caused by gram-negative pathogens either during or within 16 days following the 8 days of treatment. Macrolide-lincosamide-streptogramin (MLS) resistance was identified in 35% of *S. uberis* isolates tested and was associated with extended therapy treatment failures. Direct and indirect effects of lactation therapy in these herds were limited and suggest extended therapy of subclinical streptococcal mastitis provided limited benefits as an addition to current mastitis control practices on these farms.
The streptococcal species continue to be an important cause of mastitis in dairy cattle. The adoption of the five-point mastitis control program including milking time hygiene, risk management, and treatment practices has resulted in the successful control of some contagious pathogens including *Streptococcus agalactiae* (Hillerton and Berry, 2003). These practices seem to have been less successful in control of other (non-agalactiae) streptococcal species, commonly described as the ‘environmental’ streptococci (Hillerton and Berry, 2003). Despite 4 decades of research following development of the five-point control program, non-agalactiae streptococcal intramammary infections (IMI) remain a significant problem in modern dairy herds that have successfully implemented methods to control *S. agalactiae*, and environmental pathogens have increased in relative importance as a cause of mastitis (Hillerton and Berry, 2005).

The term ‘environmental streptococci’ has been used to describe a group of organisms, not all of the genus streptococcus, that are commonly associated with environmental sources of exposure. Hillerton and Berry (2003) provide a list of the eight most common environmental streptococci. These organisms may be isolated in pure or mixed culture either associated with intramammary infections or as milk sample contaminants. Among these species, *Streptococcus uberis* is the most frequently isolated major mastitis pathogen on many farms, and subclinical mastitis appears to be the dominant form associated with the environmental streptococci, including *S. uberis* (Bramley, 1984; Watt, 1999; Hillerton and Berry, 2003; Zadoks et al., 2003). Cases of
subclinical mastitis may be of short (i.e. transient; e.g. < 30 days) or extended (i.e. chronic; e.g. > 30 days) duration and may be characterized by the complete absence of clinical signs (i.e. fully asymptomatic), or by brief or extended asymptomatic periods with the occasional clinical episode(s) (i.e. a symptomatic period or clinical ‘flare-up’). Clinical episodes may occur at the start of or within chronic subclinical infections, or clinical cases may occur as new infection events with no evidence of an associated asymptomatic period (Dodd and Neave, 1970; Watt, 1999; Zadoks et al., 2003). Chronic quarter level infections caused by the same pathogen may display repeated clinical episodes (Watt, 1999; Zadoks et al., 2003).

Clinical mastitis is readily observed, and is frequently treated with the goal of returning milk to a normal marketable consistency (clinical cure) but often treatment is given without specific information on the cause of infection (Bramley and Dodd, 1984; Sawant et al., 2005; Pol and Ruegg, 2007). In comparison, subclinical mastitis, characterized by an elevated milk somatic cell count (SCC), frequently goes undetected or untreated by dairy producers for extended periods, and in many circumstances the reliance on observation of clinical signs will give a false impression of the extent of subclinical infections in a herd (Bramley and Dodd, 1984; Hillerton et al., 1995; Oliver et al., 2004). Increased somatic cell count is associated with reduced milk production, increased risk of antibiotic residue violations, reduced fluid milk quality, and reduced cheese yield and quality (Reneau, 1986; van Schaik et al., 2002; Santos et al., 2003). Reduced productivity and lost milk quality payments associated with subclinical mastitis represent more than half of the estimated $1.8 billion in costs attributed to mastitis for US
dairy producers annually (Ott and Novak, 2001). The association between the increased somatic cell count due to subclinical mastitis, and reduced productivity and milk quality presents a potential economic opportunity for dairy producers to implement control programs targeting subclinical mastitis. Further, the introduction of cow level SCC testing at regular intervals provides information that can be used to identify infected cows (Bramley and Dodd, 1984). A threshold of 200,000 cells per ml has been recommended to distinguish between uninfected and infected quarters or cows (Dohoo, 1991; Smith et al., 2001; Schukken et al., 2003; Hillerton and Berry, 2005), and results of sequential monthly SCC testing can be used to identify chronically infected cows (Schukken et al., 2003).

It has long been recognized that control programs that target both the reduction of new infections and the reduction of duration of infection are able to achieve the greatest success (Dodd et al., 1977). Thus the five-point mastitis plan is most effective as it controls new infection rates through use of teat hygiene, especially post milking teat disinfection, and reduces duration of infection though use of antimicrobial treatment at the end of each lactation (i.e. dry cow therapy), and to a more limited extent by treatment of clinical mastitis cases during lactation (Bramley, 1984; Bramley and Dodd, 1984). Success of this control program is likely in great part because farmers are able to implement the practices with limited increases in labor and no need to rely on diagnostic procedures (Dodd et al., 1977). The ability of this program to control streptococcal mastitis has been somewhat limited, suggesting additional measures may be required to control streptococcal mastitis (Bramley, 1984). Recent work has suggested that diagnosis
and treatment of subclinical mastitis cases during lactation may be used as an adjunct to current control practices, and under some situations may be economically beneficial (Swinkels et al., 2005b; Salat et al., 2008). However, there are limited studies evaluating the effects of lactation therapy programs targeting subclinical streptococcal mastitis in commercial dairy herds as might be applied in North America, and additional data is needed to evaluate the potential value of treating subclinical infections during lactation (Hillerton and Berry, 2003).

Measurements required to evaluate the effect of a control practice that reduces the duration of infection differ from those required to evaluate a practice that reduces the new infection rate (Dodd et al., 1977). For example, prior studies that have evaluated the effect of lactation therapy for subclinical mastitis have typically limited their analysis to the direct effects of treatment by quantifying the proportion of cure in treated individuals (Sol et al., 1997; Shephard et al., 2000; Gillespie et al., 2002; St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005; Salat et al., 2008; Sandgren et al., 2008). Most of these studies have included comparisons to untreated control groups (Shephard et al., 2000; Gillespie et al., 2002; St Rose et al., 2003; Oliver et al., 2004; Salat et al., 2008; Sandgren et al., 2008), have included other measures such as response of SCC and milk production to the treatment (Shephard et al., 2000; St Rose et al., 2003; Deluyker et al., 2005; Sandgren et al., 2008), or have evaluated host or pathogen level risk factors associated with bacteriologic cure (St Rose et al., 2003; Oliver et al., 2004; Deluyker et al., 2005; Salat et al., 2008; Sandgren et al., 2008).
In comparison, studies that have evaluated control practices designed to reduce new infection rates (e.g. studies of teat-end disinfection) require measuring new infection rates, typically by sequential interval sampling of individuals within a herd (Dodd et al., 1977; Lam et al., 1996; Lam et al., 1997b; White et al., 2006). For pathogens that may spread from cow to cow, the direct effect of a successful treatment (i.e. reduction of duration of infection) is coupled to a potential positive indirect effect of treatment, in that eliminating infections reduces transmission risk or the infection pressure (i.e. force of infection) in the population (Zadoks et al., 2002a; Barkema et al., 2006; White et al., 2006). A few prior studies have suggested this indirect component is of economic importance to the decision to implement lactation therapy programs targeting subclinical mastitis (Zadoks et al., 2002a; Swinkels et al., 2005a; Swinkels et al., 2005b). To the best of our knowledge, no negative controlled field studies of antimicrobial mastitis treatments have been designed to evaluate the indirect effect by including measurements of new infection rates in populations receiving the treatment intervention. Studies of antimicrobial treatment programs designed to measure both direct and indirect effects require the most intensive sampling schemes and rarely appear in the literature, likely as a result of their expensive and labor intensive nature, but are necessary to describe the potential overall benefits and limitations of lactation therapy targeting subclinical mastitis control.

Measurements of infection dynamics within dairy cattle populations may be improved by inclusion of methods to identify individual strains of pathogens (Luby and Middleton, 2005; Barkema et al., 2006; Zadoks and Schukken, 2006). A bacterial strain
can be defined as “an isolate or a group of bacterial isolates exhibiting characteristics that set it apart from other isolates belonging to the same species” (Zadoks and Schukken, 2006), and a number of molecular or DNA-based methods of genotyping can be used to discriminate among strains of mastitis pathogens within herds or larger populations (Phuektes et al., 2001b; Wieliczko et al., 2002; Zadoks et al., 2003). The ability to differentiate strains by molecular genotyping or fingerprinting may improve estimates of infection duration and the proportion of bacteriologic cure in studies of direct effects of mastitis therapy (Luby and Middleton, 2005; Barkema et al., 2006; Zadoks and Schukken, 2006). In addition, the identification and enumeration of strain diversity within a population receiving an intervention may provide evidence of the potential source(s) of new infections, and thus improve indirect effect estimates for mastitis treatment programs.

In an epidemiologic context, the finding of multiple bacterial strains of a single species causing mastitis in a population of cows suggests these infections arise from one or more sources of exposure that are populated with many strains. Such findings have been used to infer environmental sources of exposure for *S. uberis* mastitis in dairy herds where the environment is populated with a diverse number of *S. uberis* strain types (Phuektes et al., 2001b; Wieliczko et al., 2002; Zadoks et al., 2003; Pullinger et al., 2006; Pullinger et al., 2007). In comparison, the finding of a limited number of strains within a herd experiencing a mastitis outbreak over a defined time period may be consistent with cow to cow transmission as has also been suggested for *S. uberis* in some herds or management scenarios (Phuektes et al., 2001b; Zadoks et al., 2001a). In these later cases
it is possible that clusters of infection with a limited number of strains in space or time can also arise from a common environmental source populated with a dominant strain, or from the enhanced virulence of some individual environmental strains (Phuektes et al., 2001b; Munoz et al., 2007).

The objective of this negative controlled clinical field trial was to evaluate the direct and indirect effects of a diagnosis driven treatment program targeting chronic subclinical mastitis caused by *Streptococcus* spp. We used PCR-based methods to identify the streptococcal species causing intramammary infections, and applied random amplified polymorphic DNA (RAPD) methods to discriminate among *S. uberis* strain types. For *S. uberis*, *S. dysgalactiae* individually, and the other gram-positive catalase-negative (GPCN) cocci collectively, we determined direct effects of the treatment program by measuring duration of infection and proportion of cure in treatment and control groups. In addition, we evaluated species and strain specific rates of new infection in treatment and control groups as a measure of potential indirect effects of treatment.

Materials and Methods

*Study design*

A negative-controlled treatment trial was conducted for 13 months on 2 commercial dairy herds with free-stall housing and cows milked in parlor systems. Herds
1 and 2 were located in New York, and Vermont, USA, respectively. Enrolled cows within each herd were assigned to one of two pens, with pens randomly allocated to treatment and control groups on each farm. In the month preceding the start of the study, cows were systematically assigned to pens based on odd or even identification numbers. Cows that calved during the study were assigned to either the treatment or negative control groups based on odd-even identification number. Within farms, no differences in mean parity, days in milk, or somatic cell count, were found among treatment and control groups at the start of the study by using this assignment method.

The population of cows in the treatment and control pens was a dynamic group, with entries and exits of cows from these pens following normal management cycles. Generally, early lactation cows entered the study pens after being housed in a ‘fresh cow group’ for approximately 14 days postpartum. Dates of all entries and exits to and from treatment or control pens were recorded for each cow. The herds were milked 3 times per day, and pens of cows within each herd were milked in the following order at each milking session: fresh cow group, study treatment group, study control group, additional groups not enrolled in study. The milking system was washed and sanitized between each of their 3 daily milking sessions. There was no rinsing or disinfection of milking units after milking individual cows during a milking session, and neither farm used a back-flushing system.

The farms applied accepted mastitis control practices to all cows including use of iodine based (0.5 to 1 %) pre- and post-milking teat disinfectant solutions, and blanket use of commercially available dry-cow therapy products (herd 1, cephapirin benzathine;
herd 2, cloxacillin benzathine). The use of somatic cell count or bacteriologic culture data to segregate chronically infected cows was not practiced on either farm. The use of bacteriologic culture to aid clinical mastitis treatment decisions was not practiced on either farm. No diagnosis and treatment of subclinical mastitis was practiced on either farm prior to the start of the study. Products used to treat clinical mastitis included commercially available intramammary formulations of amoxicillin, cepapirin sodium, or pirlimycin hydrochloride, on both farms. Lactating cows in herd 1 were bedded on mattresses with sawdust bedding, while in herd 2 lactating cow stalls were deep-bedded with sand. Stocking density of study pens on both farms exceeded number of stalls by 18 to 20%. In both herds dry cows were loose-housed in a separate covered facility on a chopped straw bedded pack. Herd owners were financially compensated for participation. The study was conducted with approval of the University of Vermont Institutional Animal Care and Use Committee (IACUC).

Milk sample collection and bacteriologic analysis

Composite milk samples were collected monthly by DHIA technicians and processed through the regional commercial testing laboratories for SCC testing. Individual quarter milk samples were collected for microbiologic analysis from all cows in control and treatment groups at the start of the study, at monthly intervals for the duration of the study, and at the end of the study. These quarter samples were collected within 3 days of DHIA monthly composite sample collection. Quarter milk samples were also collected from all cows within 3 days following parturition (fresh sample),
immediately following identification of clinical mastitis (clinical pre-treatment sample),
immediately prior to treatment of subclinical mastitis (subclinical pre-treatment sample),
at any time when cows were added to or removed from the study pens for greater than 24
hours (entry/exit sample), immediately prior to exit from the herd (cull sample), and at 7,
14, 21, and 28 (± 2) days following cessation of any antibiotic therapy (clinical or
subclinical post-treatment samples). Monthly sample collection was conducted by
trained field technicians, while farm personnel were trained to collect all additional
samples following established aseptic methods (Hogan et al., 1999). Samples collected
by farm personnel were immediately stored frozen at -20°C and transported frozen to the
microbiology laboratory at two-week intervals. Monthly samples were held on ice
immediately following collection and during transport to the laboratory, and were stored
frozen at -20°C for 24 – 72 hours. All samples were thawed over-night under
refrigeration and aerobic bacteriologic culture and interpretation of results were
performed according to established guidelines (Hogan et al., 1999). Briefly, 10 uL per
sample was plated on to tryptic soy agar (TSA) containing 5% sheep blood and 0.1%
esculin. Samples with >3 morphologically distinct colony types were considered
contaminated and eliminated from analysis. For all morphologically distinct colony types
in uncontaminated samples, the number of colony forming units (cfu) per 0.01 ml was
recorded as a one of four categories: 1 to 4, 5 to 9, 10 to 49, or ≥ 50 cfu (Dingwell et al.,
2003). Representative colonies of GPCN cocci were identified based on colony
 morphology, esculin hydrolysis, Gram staining, and catalase testing. Isolates were
transferred to a TSA plate for isolation and individual isolates were transferred to broth culture and stored at -80°C.

**Treatment program**

The intent of the study was to model a treatment program that might be applied on commercial farms where an elevated SCC is used to trigger one or more bacteriologic cultures and culture results are used to trigger a treatment decision. In this study, a prolonged elevated SCC and bacteriologic culture information were used to trigger a decision to treat a quarter with mastitis caused by CPCN cocci using a commercially available intramammary formulation of a narrow spectrum antimicrobial. For this purpose, the monthly quarter milk sample taken by research personnel within 3 days of SCC testing was defined as the ‘enrollment’ sample, and was considered a proxy for a farmer collected milk sample taken following receipt of monthly SCC test results. Cows assigned to the treatment group that had an elevated composite SCC (≥200,000 cells/ml) in the current month, plus an elevated SCC in one of the two previous months and a positive culture due to CPCN cocci in the current monthly enrollment sample were eligible for extended pirlimycin therapy during that month. Cows with the most recent monthly enrollment sample where a subclinical IMI due to a gram-negative pathogen was diagnosed were not enrolled for therapy because of the limited antimicrobial spectrum of pirlimycin. Cows with a clinical mastitis event within the previous 14 days of the enrollment sample were not eligible for extended therapy. Cows that had received extended therapy but had not cured were not re-enrolled for extended therapy in the
current lactation. No additional covariates that might influence treatment success (e.g. cow lactation number, number of infected quarters, duration of infection, or antimicrobial susceptibility phenotype) were used to select cows for treatment. Treated cows on both farms were segregated in a ‘treated-cow’ pen during treatment and subsequent milk withhold periods. A maximum of approximately 6 cows could be enrolled for extended therapy at any particular time due to the small pen size and limited number of available stalls in the treated-cow holding pen on each farm. Cows in the control group were monitored for treatment eligibility based on monthly SCC and bacteriology but received neither extended therapy, nor an excipient infusion. All lactating quarters of an enrolled cow were infused once daily with a commercially available intramammary formulation of the lincosamide antibiotic pirlimycin hydrochloride (Pirsue™, Pfizer Animal Health, New York) at the labeled daily dosage (50 mg) for an extended (8 days) duration of therapy (extended therapy) (Gillespie et al., 2002; Oliver et al., 2003; Deluyker et al., 2005). Farmers collected quarter milk samples from enrolled cows immediately prior to the initial extended therapy treatment. Cows experiencing clinical mastitis in either group were treated using the established practices on the participating farms and records were maintained on the type and duration of therapy. In general, antimicrobials used for intramammary treatment of clinical mastitis were restricted to commercially available formulations, including amoxicillin, cephapirin sodium, and pirlimycin hydrochloride on farm 1, and amoxicillin and pirlimycin hydrochloride on farm 2. There was a 3-month ‘pre-intervention’ observation period at the start of the study prior to a 10 month intervention period. All data were collected during both the initial 3 month observation
and the subsequent 10 month intervention period. Figure 4.1 provides a summary of the study design and sample collection procedures.

<table>
<thead>
<tr>
<th>Group</th>
<th>Procedures</th>
<th>Month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>Monthly Composite SCC – cows with SCC &gt; 200,000 cells/ml in current month and at least 1 of 2 previous months identified as having chronic subclinical mastitis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monthly Quarter Milk Bacteriologic Culture – samples taken within 3 days of composite SCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extended therapy of subclinical mastitis after month 3 cows identified with chronic subclinical mastitis and bacteriologic culture positive for GPCN corel identified as eligible for extended therapy and treatment initiated</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Monthly Composite SCC – cows with SCC &gt; 200,000 cells/ml in current month and at least 1 of 2 previous months identified as having chronic subclinical mastitis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monthly Quarter Milk Bacteriologic Culture – samples taken within 3 days of composite SCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Data collection only – no extended therapy treatments of cows identified with chronic subclinical mastitis</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.1 Study design and sample collection schedule for a subclinical mastitis treatment trial

**Infection status**

Infection status was defined at two levels. First, as described in the previous section, using monthly SCC data to model treatment decisions as might be applied on commercial farms, chronic subclinical mastitis was defined as SCC ≥ 200,000 cells/ml for at least 2 of the past 3 serial monthly composite milk samples (Schukken et al., 2003). After identifying cows with chronic subclinical mastitis each month, the current month’s
bacteriologic results were used to identify cows that were eligible for treatment in that month. Extended therapy treatments were limited to cows in the treatment group with at least one quarter with a presumptive infection caused by GPCN cocci, and no concurrent isolation of a gram-negative pathogen, or clinical mastitis episode within the past 14 days. This assumes that whole herd serial monthly bacteriologic culture is not cost effective for routine use on commercial farms, but that monthly SCC test data is frequently available which can be used to select individual cows for culture and possible treatment based on culture results (Schukken et al., 2003).

Second, IMI status was defined based on sequential culture results as previously described by Zadoks, et al. (2002a). This data was used retrospectively to determine monthly infection prevalence and to evaluate the direct (bacteriologic cure proportions) and indirect (new infection rates) effects of the treatment program. These serial culture data were used to define the IMI status of individual quarters independent of the composite SCC results, and IMI status was not used as a trigger for extended therapy treatment of subclinical mastitis. Based on the previously published definitions of Zadoks, et al. (2002a), individual quarters were categorized as having an IMI when a 10 uL milk sample was positive on bacteriologic culture based on at least one of the following criteria: 1) ≥1000 cfu/ml from a single sample, 2) ≥500 cfu/ml from two out of three consecutive samples, 3) ≥100 cfu/ml from 3 of 3 consecutive samples, or 4) ≥100 cfu/ml from a clinical sample. Samples with >3 morphologically distinct colony types were considered contaminated and eliminated from analysis. Samples with bacteria isolated that did not meet the above criteria were defined as incidental isolation events.
and were presumed to be unimportant isolates possibly associated with transient or extended colonization of teat skin or orifices or with fecal contamination of these surfaces or workers’ hands (Bramley, 1984; Erskine and Eberhart, 1988; Hillerton and Berry, 2003; Zadoks et al., 2003).

At each sampling point, an IMI was defined as either subclinical (i.e. asymptomatic) or clinical (symptomatic). It was recognized that an IMI first observed as a subclinical case may show sporadic or occasional episodes of clinical mastitis (clinical ‘flare-up’), and that an IMI first observed as a clinical case event may persist as a subclinical mastitis. A clinical mastitis case was defined as an abnormality in appearance or consistency of milk, with or without either local and/or systemic signs associated with infection. Clinical mastitis severity was not categorized. A new clinical mastitis case or event was identified at the quarter level after that quarter was observed free of clinical signs for $\geq 14$ days or if clinical mastitis occurred within 14 days of a previous case but was caused by a different pathogen species or strain. An individual quarter was defined as having a subclinical IMI, when meeting any one of the first three bacteriologic criteria, and having no occurrence of clinical symptoms within the past 14 days. Thus for the purpose of this study, and consistent with established epidemiologic concepts (Dodd and Neave, 1970), a single persistent infection could be characterized by extended periods of subclinical mastitis punctuated by one or more clinical episodes.

By establishing two parallel definitions, it was recognized that a cow with a quarter level IMI may have either a high or low SCC and the IMI may be either persistent (chronic) or of short duration (transient), making the IMI categories used for analysis of
infection dynamics distinct from the SCC based subclinical mastitis categories used for initiating treatment decisions. A chronic subclinical IMI was defined as a quarter with an IMI caused by the same species or strain on at least 2 of 3 sequential monthly (28 day) sample intervals. A transient subclinical IMI was defined as an IMI observed from a single isolation event (e.g. culture of $\geq 1000$ cfu/ml from a single sample), or two isolation events less than 28 days apart. It was possible for a cow with chronic subclinical mastitis (based on SCC results) to be enrolled for extended therapy following isolation of GPCN cocci from the enrollment sample but in retrospect to be defined as not having an IMI based on results that include a culture negative pretreatment sample. These events were recorded as ‘false positive’ treatments and were not included in analysis of IMI cure proportions.

A quarter with a subclinical IMI was defined as spontaneously cured when the quarter was negative for the same pathogen or strain on 2 subsequent consecutive samples (generally at 28 and 56 days following the last positive sample). This definition was used to limit the number of errors in defining a cure due to the potential of observing a single false negative culture result on serial samples (Morant et al., 1988). A subclinical IMI was defined as cured following extended therapy when culture negative for the pre-treatment species or strain on 4 of 4 post treatment samples taken at 7 (± 2) day intervals (Gillespie et al., 2002), accounting for the possibility of post-treatment false negative culture results. A clinical IMI was defined as a bacteriological cure following treatment when culture negative for the pre-treatment species or strain on 4 of 4 post treatment samples taken at 7 (± 2) day intervals. The infection status following either spontaneous
cure or therapeutic cure was monitored for the duration of the study or until an animal
exited the study population. Data on infection status and cure was recorded for all aerobic
bacterial species identified during the trial. A re-infection of a quarter that was
previously defined as cured followed the above IMI definitions, after the observation of
at least two bacteriologic negative samples.

**Duration of infection**

Duration of infection was calculated based on mid-point estimation method
previously described by Zadoks et al. (2003), where start of the IMI was defined as the
middle of the time interval between a negative culture and the first positive culture event,
and end of the IMI was defined as the middle of the time interval between the last
positive culture and the first negative culture event for a quarter defined as cured.

Quarters that entered the study infected, either at the start of the study or at the start of
lactation, were considered left censored IMI and the infection start date was the date of
first positive culture at time of enrollment. Infected quarters that exited the study were
considered right censored and the IMI end date was the date of exit or the end date of the
study for those lactating quarters still infected on the last monthly sample date. Quarter
days infected (IMI prevalence days) in the study pens were calculated based on recorded
dates of cow entry and exit from the study pens for all lactating quarters. Quarter days
susceptible (i.e. days at risk for new IMI among uninfected quarters) were calculated
based on recorded dates of cow entry and exit from the study pens accounting for the date
of changes in quarter infection status during each interval. Non-lactating quarters in
lactating cows were identified with the date of milking cessation of the individual quarters (i.e. quarter ‘culling’) so that data on infected or susceptible quarter days accounted for any ‘blind’ or culled quarters within lactating cows.

*Species identification and S. uberis strain typing*

Pure subcultures were obtained for species identification from selected isolates representing all IMI caused by GPCN cocci. Isolates were selected from early, middle, and late time points. In addition, pre- and post-treatment isolates were selected for all cows receiving lactation therapy. For initial identification, *Streptococcus uberis* and *Streptococcus dysgalactiae* were identified by multiplex PCR directly from bacterial suspensions (Martineau et al., 2000) using established methods (Phuektes et al., 2001a) with slight modifications. Briefly 3-5 colonies from a pure subculture were suspended in 100 uL TE, and 1 uL of the bacterial suspension was transferred to a 50 uL final volume containing PCR reagents and *S. uberis* and *S. dysgalactiae* primers previously described by Phuektes et al. (2001a). Cell lysis was by extended heating during a prolonged initial denaturing step of 94°C for 15 min at the start of each thermocycler program, with amplification performed by 36 cycles of 95°C for 1 minute, 50°C for 30 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 5 minutes. PCR product was resolved by electrophoresis through a 1.5% or 2% agarose gel stained with ethidium bromide and visualized under UV light. Species identity was based on amplification product size estimated by comparison with a 50-bp molecular size ladder and amplification products run in parallel for *S. uberis* and *S. dysgalactiae* control strains.
Absence of an amplification product suggested identification of GPCN cocci other than *S. uberis* or *S. dysgalactiae*. Selected isolates that were negative for *S. uberis* or *S. dysgalactiae* PCR were identified by amplification and sequencing of a 740 bp fragment of the *rpoB* gene (encoding the beta subunit of RNA polymerase) using previously described primers and methods (Drancourt et al., 2004).

Isolates initially identified as *S. uberis* were confirmed by a second independent uni-plex PCR reaction with DNA template prepared from pure subculture by established methods (Furrer et al., 1991). Strain typing of *S. uberis* isolates was performed by RAPD as previously described (Schmitt-Van de Leemput and Zadoks, 2007). RAPD type was considered to be the same for isolates having the same number and size of DNA fragments, regardless of band intensity. Individual RAPD types were designated with an arbitrary numeric code, preceded by a letter designation for each of the two farms. Two independent observers visually evaluated banding patterns to discriminate among RAPD types, and any discrepancies in RAPD type classification were resolved by joint re-evaluation. Comparisons of banding patterns were limited to within an individual gel where sets of up to 18 isolates were processed simultaneously and run on a single gel for visualization. Sufficient gels were run to make within quarter, within cow, and within farm comparisons of isolates in order to discriminate among potential strain differences at each of these levels.
Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined retrospectively by broth dilution and disc diffusion methods for pretreatment isolates of IMI receiving extended pirlimycin therapy. For each IMI, susceptibility test results were determined for a minimum of 2 pre-treatment isolates obtained at separate time-points (e.g. the enrollment sample isolate and the subclinical pre-treatment sample isolate).

Minimum inhibitory concentrations were determined by broth microdilution methods using commercially prepared micro-titer plates (Sensititre, Trek Diagnostics, Cleveland, Ohio), and performed in accordance with Clinical Laboratory Standards Institute guidelines (2008). Briefly, bacterial suspensions in sterile demineralized water standardized to a 0.5 McFarland were made from pure subcultures and used to inoculate commercially prepared cation adjusted Mueller-Hinton broth with TES buffer (Trek Diagnostics, Cleveland, Ohio) per manufacturer instructions. Aliquots (50 uL) of this broth were immediately dispensed into wells of 96-well plates containing two-fold serial dilutions of ampicillin (0.12 to 8 ug/ml), penicillin (0.12 to 8 ug/ml), oxacillin (2 to 4 ug/ml), erythromycin (0.25 to 4 ug/ml), pirlimycin (0.5 to 4 ug/ml), penicillin/novobiocin (1/2 to 8/16 ug/ml), tetracycline (1 to 8 ug/ml), cephalothin (2 to 16 ug/ml), ceftiofur (0.5 to 4 ug/ml), and sulphadimethoxine (32 to 256 ug/ml). Plates were incubated for 18 to 24 hours at 37°C and MICs were determined visually using an inverted magnifying mirror. Positive growth controls were included in each plate. Quality control organisms Staphylococcus aureus (ATCC strain 29213) and Enterococcus faecalis (ATCC strain 29212) were run in parallel with each series of
isolates tested. Inoculum purity controls were examined by streaking aliquots of the inoculated Mueller-Hinton (M-H) broth to TSA with 5% sheep blood and incubating for 48 hours at 37°C to detect mixed cultures due to contamination. Testing was repeated from a pure subculture if a mixed culture was suspected.

Agar disc diffusion susceptibility testing was conducted for ampicillin (10 ug), penicillin (10 IU), erythromycin (15 ug), pirlimycin (2 ug), penicillin/novobiocin (10 IU / 30 ug), tetracycline (30 ug), cephalothin (30 ug), ceftiofur (30 ug), clindamycin (2 ug) and vancomycin (3 ug). Colonies from pure subcultures were used to inoculate tryptic soy broth (TSB), which was incubated for 2 to 12 hours and when necessary diluted with saline to achieve a 0.5 McFarland turbidity suspension. A sterile cotton swab was used to streak the suspension on Mueller-Hinton agar with 5% sheep blood to create a confluent bacterial lawn. Antimicrobial discs were applied within 15 minutes of streaking the lawn and zone diameters determined after plates were incubated for 18-20 hours at 37°C. Inoculum purity controls were performed from the TSB as described for M-H broth. For isolates demonstrating erythromycin resistance either by disc diffusion or broth dilution but appearing susceptible to either clindamycin or pirlimycin the D-test was performed in accordance with recommended methods to identify inducible lincosamide resistance (Steward et al., 2005; Schmitt-Van de Leemput and Zadoks, 2007).

For each testing method, isolates were categorized as susceptible, intermediate or resistant based on criteria established for streptococci other than Streptococcus pneumoniae, for all antimicrobials examined, except for pirlimycin and
penicillin/novobiocin which were categorized based on criteria for streptococci causing bovine mastitis (CSLI, 2008).

Statistical methods

All statistical analysis was conducted using SAS version 9 (SAS Institute, Inc., Cary, NC, USA). The effect of treatment on the natural logarithm of duration (LnIMIDUR) was tested in a generalized linear model including the effect of streptococcus species, farm and the interactions between farm and treatment group and species and treatment group. The effects of treatment on duration were also explored using a data set containing only IMI defined as chronic. The hypothesis that treatment affected duration of *S. uberis* and *S. dysgalactiae* infection was tested using the Log-rank test to compare the Kaplan-Meier survival function for treated and control groups, while controlling for the effect of farm and species, with mean [standard deviation (sd)], and median [95% confidence interval (CI)] estimates reported.

Prevalence (number of quarters days infected, $P_T$) and incidence(number of new IMI, $I_N$) of *S. uberis or S. dysgalactiae* IMI were analyzed as outcomes using generalized linear models (PROC GENMOD, SAS) and model checking included examination of deviance and Pearson Chi-square for goodness of fit (Lam et al., 1996; Zadoks et al., 2002a). Evidence of overdispersion was adjusted using Pearson Chi-square estimates divided by the degrees of freedom (Pscale option). Binomial, Poisson, or Negative Binomial error distributions were selected for final models after comparisons for goodness of fit. Treatment group (*Group*) and time period (2 categories, pre-intervention
versus intervention periods) were included as dependent variables. In the final
prevalence model, the impact of treatment group on the prevalence in each interval was
modeled assuming a negative binomial distribution, a log link, and an offset of the total
number of quarter days,

\[ \epsilon \left[ \ln(P_T) \right] = \text{intcpt} + \text{Group} + \text{Timep} + \text{Group} * \text{Timep} + \ln(N) \]  \[4.1\],

where \( \epsilon \) = expected value and \( \ln(N) \) = offset. Comparison of least square means IMI
quarter-days prevalence (\( P_T \)) between treatment and control groups and across
observation and intervention time periods was conducted separately for each farm using
model 4.1 and accounting for multiple comparisons with Bonferroni methods.

The impact of treatment on incidence in each interval was estimated in a Poisson
regression model, with a Poisson error distribution, a log link, and an offset of the
number of quarter days susceptible for new IMI (\( S \)),

\[ \epsilon \left[ \ln(I_s) \right] = \text{intcpt} + \text{Group} + \text{Timep} + \text{Group} * \text{Timep} + \ln(S) \]  \[4.2\].

Since prevalence was affected by treatment, prevalence was not included as a covariate in
model 4.2.

Results

On farms 1 and 2 there were a monthly average of 319 and 346 lactating cows,
respectively. The monthly average number of cows in treatment and control pens at the
time of monthly DHIA SCC testing was 95 and 98, respectively, on farm 1, and, 91 and
89, respectively on farm 2. On farm 1 milk production averaged 69 and 72 lbs per day, while on farm 2 production averaged 93 and 91 pounds per day, in treatment and control pens, respectively. The mean lactation number of cows in control pens and treatment pens did not differ on either farm, and the overall mean lactation number of cows enrolled in the study on farm 1 and 2 was 2.44 and 3.05, respectively.

A total of 31,761 quarter milk samples were collected from the two farms. On farm 1 there were 14,467 quarter samples taken from 3675 sample events from 385 cows, with 209 (1.4%) samples identified as contaminated leaving 14258 quarter samples for analysis. Of these, GPCN cocci were isolated from 1313 (9.2%) samples. On farm two there were 17,294 quarter samples taken from 4387 sample events from 580 cows, with 787 (4.6%) samples identified as contaminated leaving 16507 quarter samples for analysis. Of these, GPCN cocci were isolated from 1340 (8.1%) samples.

Intramammary infection cure proportions in treated and control groups

Farm 1. On farm 1, eight *Streptococcus* spp. IMI in 8 quarters of 8 cows were treated by extended therapy. Six (75%) IMI were defined as cures including 3 of 3 *S. dysgalactiae*, 0 of 2 *S. uberis*, and 3 of 3 other GPCN cocci (*S. parauberis*, *S. pluranimalium*, and one species not determined). The frequency of IMI events including treatments within each monthly interval for *S. dysgalactiae* and *S. uberis* for treatment and control groups on farm 1 are shown in tables 4.1 and 4.2.

A total of 16 *S. dysgalactiae* IMI in 14 quarters of 12 cows, and 12 *S. uberis* IMI in 12 quarters of 6 cows, were identified in the treatment pen on farm 1. Of the *S.
dysgalactiae IMI in the treatment pen, 3 IMI cured following extended therapy of subclinical mastitis and 2 cured following treatment of a clinical episode. Of the 6 chronic IMI that received no treatment of clinical or subclinical mastitis in this pen, 2 cured spontaneously. No S. uberis IMI were defined as cures following extended therapy of subclinical mastitis, however both quarters subsequently cured spontaneously. Bacteriologic cure was observed for 2 of 7 S. uberis IMI that were treated because of clinical episodes. In the treatment pen on farm 1 there were 6 S. uberis IMI that were not treated with extended therapy or for clinical mastitis, including 3 transient IMI that cured spontaneously, and 3 chronic IMI that left the study population infected.

Seven S. dysgalactiae IMI in 6 quarters of 6 cows, and 5 S. uberis IMI in 5 quarters of 5 cows, were identified in the control pen on this farm. Of the S. dysgalactiae IMI in the control pen, 2 cured following treatment of a clinical episode, and 3 of 5 chronic subclinical IMI cured spontaneously. Five S. uberis IMI in 5 quarters of 5 cows were identified in the control pen on farm 1. Two of 3 clinical S. uberis episodes in 2 quarter level IMI cured following treatment in the control pen. In the control pen on farm 1 there were 2 spontaneous cures of 3 chronic S. uberis IMI that were not treated for clinical mastitis (tables 4.1 and 4.2).

Overall, the proportion of chronic subclinical IMI that cured spontaneously on farm 1 (including IMI in the treatment and control pens that did not receive any lactation therapy) was 45% (5/11) for S. dysgalactiae and 33% (2/6) for S. uberis. Streptococci were cultured from 22 clinical episodes in 16 quarters of 13 cows in herd 1 caused by S. uberis (n=10), S. dysgalactiae (n=11), and other GPCN cocci (not typed n=1). Cure
following clinical therapy was observed for 8 (36%) of these cases including, 4 *S. dysgalactiae* and 4 *S. uberis* IMI.

**Farm 2.** On farm 2, eighteen GPCN cocci IMI in 18 quarters of 15 cows were treated by extended therapy. One cow was culled due to clinical mastitis caused by *Escherichia coli* 3 days following the eighth day of treatment and was considered a treatment failure in the analysis of cure proportions. The frequency of IMI events including treatments within each monthly interval for *S. dysgalactiae* and *S. uberis* for treatment and control groups on farm 2 are shown in tables 4.3 and 4.4.

Three *S. dysgalactiae* IMI in 3 quarters of 3 cows, and 41 *S. uberis* IMI in 21 quarters of 17 cows, were identified in the treatment pen on farm 2. Of the *S. dysgalactiae* IMI in the treatment pen, one IMI each cured following extended therapy of subclinical mastitis and treatment of clinical IMI, and one spontaneously cured. Of the *S. uberis* IMI in the treatment pen, 11 of 15 cured following extended therapy of subclinical mastitis, while 2 of 4 IMI cured following a clinical episode. Two of the 4 chronic subclinical IMI that did not cure following extended therapy subsequently cured spontaneously. In the treatment pen on farm 2 there were 16 spontaneous cures of 22 *S. uberis* IMI that were not treated with extended therapy or for clinical mastitis, including 7 transient IMI, and 15 chronic IMI.

Six *S. dysgalactiae* IMI in 5 quarters of 5 cows, and 32 *S. uberis* IMI in 22 quarters of 14 cows, were identified in the control pen on farm 2. Of the *S. dysgalactiae* IMI in the control pen, two IMI cured following treatment of a clinical episode, and 2 of 4 chronic subclinical IMI cured spontaneously. Five of 11 clinical *S. uberis* episodes in 7
quarter level IMI cured following treatment in the control pen on farm 2. In the control pen on farm 2 there were 13 spontaneous cures of 25 *S. uberis* IMI that were not treated for clinical mastitis, including 3 transient IMI, and 22 chronic IMI (table 4.3 and 4.4).

Overall, the proportion of chronic subclinical IMI that cured spontaneously on farm 2 (including IMI in the treatment and control pens that did not receive any lactation therapy) was 54% (19/35) for *S. uberis* and 60% (3/5) for *S. dysgalactiae*. Streptococci were cultured from 23 clinical episodes in 19 quarters of 17 cows in herd 2 caused by *S. uberis* (n=15), *S. dysgalactiae* (n=5), *Lactococcus lactis* (n=1), and *Enterococcus faecium* (n=2). Cure following clinical therapy was observed for 10 (43%) of these episodes including, 3 *S. dysgalactiae* and 7 *S. uberis* IMI.

The overall proportion of quarters with chronic subclinical IMI that cured following extended therapy was 65% (11/17) and 100% (4/4) for *S. uberis* and *S. dysgalactiae*, respectively; while 51% (21/41) and 50% (8/16) of chronic subclinical IMI spontaneously cured for *S. uberis* and *S. dysgalactiae*, respectively. In a logistic model, extended lactation therapy was not a significant predictor of the probability of cure among cases of chronic subclinical mastitis, accounting for the effects of IMI species (*S. uberis* vs. *S. dysgalactiae*), farm, and the 2 way interaction between farm and species.
Table 4.1 Frequency of *Streptococcus uberis* intramammary infections in treatment and control groups on Farm 1, stratified by epidemiologic categories.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Streptococcus uberis</th>
<th>Sample Interval (month)</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>Herd 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment Group</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Left censored 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New IMI 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total IMI interval</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EX eligible 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Suspected 4</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Suspected clinical cure 6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Right censored 7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic IMI remaining at end of interval</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>number clinical events</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Control Group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment Group</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Left censored 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New IMI 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total IMI interval</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>EX eligible 5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Suspected 4</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Suspected clinical cure 6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Right censored 7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic IMI remaining at end of interval</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>number clinical events</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. Left censored, quarter entering pen with existing intramammary infection (IMI).
2. New IMI, quarter previously identified as negative in pen.
3. Eligible for extended therapy based on low level 30G ≤ 100,000 cells/ml and current positive culture for *Streptococcus uberis*.
4. Treated once daily with intramuscular injection of penicillin G (20,000 units) for 8 days.
5. Post-treatment cure defined as negative for pre-treatment strain type on 4 of 4 samples taken at 7 (± 2) day intervals.
6. Sponaneous cure defined as IMI found negative for previous strain on 2 of 2 samples taken at least 25 days apart.
7. Right censored, quarter exiting the pen with existing IMI.

*Number of asterisk indicates number of clinical events associated with either an existing IMI (flare-up) or a new IMI clinical at start.
Table 4.2 Frequency of *Streptococcus dysgalactiae* intramammary infections in treatment and control groups on Farm 1, stratified by epidemiologic categories.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sample Interval (month)</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus dysgalactiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left censored</td>
<td>1  1  0  1  0  0  0  0  0  0  0  0  0  4</td>
<td></td>
</tr>
<tr>
<td>New IMI</td>
<td>0  1  0  0  0  0  1*  0  1*  0  2*  2  1*  3*  1  12</td>
<td></td>
</tr>
<tr>
<td>Total IMI</td>
<td>1  3  3  3*  3  2  1  1  2  2  2  4**  4  16</td>
<td></td>
</tr>
<tr>
<td>SX eligible</td>
<td>1  2  2  2  2  2  2  1  0  0  0  1  0  0  2  5</td>
<td></td>
</tr>
<tr>
<td>Su treated</td>
<td>na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  0  0  3</td>
<td></td>
</tr>
<tr>
<td>Su cure</td>
<td>na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  3  3  3</td>
<td></td>
</tr>
<tr>
<td>Spon. cure</td>
<td>0  0  0  0  1  0  1  1  0  0  0  1  0  0  1  0  4</td>
<td></td>
</tr>
<tr>
<td>Clinical cure</td>
<td>0  0  0  0  0  0  0  0  0  0  1  0  0  1  0  2</td>
<td></td>
</tr>
<tr>
<td>Right censored</td>
<td>0  0  1  0  0  0  0  0  0  0  1  0  1  0  1  7</td>
<td></td>
</tr>
<tr>
<td>Chronic IMI</td>
<td>1  2  2  3  1  1  0  0  0  1  1  3  3  3</td>
<td></td>
</tr>
<tr>
<td>remaining at end of interval</td>
<td>0  0  0  1  0  1  0  1  1  0  2  3  0  9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control Group</th>
<th>Sample Interval (month)</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus dysgalactiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left censored</td>
<td>1  0  0  0  0  0  0  0  0  1  0  0  0  0  2</td>
<td></td>
</tr>
<tr>
<td>New IMI</td>
<td>0  0  0  0  0  0  0  0  0  0  0  0  2*  0  0  2*  1  5</td>
<td></td>
</tr>
<tr>
<td>Total IMI</td>
<td>1  1  1  0  0  0  0  0  0  3  2  0  1  2  7</td>
<td></td>
</tr>
<tr>
<td>SX eligible</td>
<td>1  1  1  0  0  0  0  0  0  1  0  0  1  3</td>
<td></td>
</tr>
<tr>
<td>Su treated</td>
<td>na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na</td>
<td></td>
</tr>
<tr>
<td>Su cure</td>
<td>na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na  na</td>
<td></td>
</tr>
<tr>
<td>Spon. cure</td>
<td>0  0  1  0  0  0  0  0  0  0  2  0  0  0  5</td>
<td></td>
</tr>
<tr>
<td>Clinical cure</td>
<td>0  0  0  0  0  0  0  0  0  0  1  0  0  1  0  2</td>
<td></td>
</tr>
<tr>
<td>Right censored</td>
<td>0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  2</td>
<td></td>
</tr>
<tr>
<td>Chronic IMI</td>
<td>1  1  0  0  0  0  0  0  0  2  0  0  1  2  2</td>
<td></td>
</tr>
<tr>
<td>remaining at end of interval</td>
<td>0  0  0  0  0  0  0  0  0  1  0  0  1  0  2</td>
<td></td>
</tr>
</tbody>
</table>

1. Left censored, quarters entering pen with existing intramammary infection (IMI).
2. New IMI, quarters previously identified as negative in pen.
3. SX eligible for extended therapy based on cow level SCC (100,000 cells/ml) and current positive culture for streptococcal species.
4. Treated twice daily with intramammary infusion of penicillin and hydrocortisone (10 mg) for eight days.
5. Post-treatment cure defined as negative for penicillin and streptococcal species on four of four samples taken at 7 to 14 day intervals.
6. Sponaneous cure defined as IMI found negative for previous strain on two of two samples taken at least 30 days apart.
7. Right censored, quarters exiting the pen with existing IMI.

* Number of asterisk indicates number of clinical events associated with either an existing IMI (flare-up) or a new IMI clinical at start.
Table 4.3 Frequency of *Streptococcus uberis* intramammary infections in treatment and control groups on Farm 2, stratified by epidemiologic categories.

<table>
<thead>
<tr>
<th>Herd 1</th>
<th>Streptococcus uberis</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Group</td>
<td>Left censored 1</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>New IMI 2</td>
<td>1*</td>
<td>3</td>
<td>1</td>
<td>3*</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Total IMI interval</td>
<td>13</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>15</td>
<td>10*</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>SX eligible 3</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>12</td>
<td>12</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>SX treated 4</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>SX cure 5</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Spont. cure 6</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Clinical cure 7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Right censored 1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Chronic IMI remaining at end of interval</td>
<td>8</td>
<td>12</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>number clinical events</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Control Group</td>
<td>Left censored 1</td>
<td>11</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>New IMI 2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1*</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Total IMI interval</td>
<td>12*</td>
<td>12***</td>
<td>9*</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>6*</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>5**</td>
<td>4**</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SX eligible 3</td>
<td>4</td>
<td>12</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>SX treated 4</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>SX cure 5</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Spont. cure 6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Clinical cure 7</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Right censored 1</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Chronic IMI remaining at end of interval</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>number clinical events</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

1. Left censored, quarters entering pen with existing intramammary infection (IMI).
2. New IMI, quarters previously identified as negative in pen.
3. Eligible for extended therapy based on cow level SCC: >200,000 cells/mL and current positive culture for *Streptococcus* species.
4. Treated once daily with intramammary infusion of pirlimycin hydrochloride (30 mg) for eight days.
5. Post-treatment test cure defined as negative for pre-treatment strain type on 4 of 5 sample taken at 7 (±2) day intervals.
6. Spontaneous cure defined as an IMI found negative for previous strain on 2 of 2 samples taken at least 28 days apart.
7. Right censored, quarters exiting the pen with existing IMI.

*Number of asterisk indicates number of clinical events associated with either an existing IMI (flare-up) or a new IMI clinical at start.
Table 4.4 Frequency of *Streptococcus dysgalactiae* intramammary infections in treatment and control groups on Farm 2, stratified by epidemiologic categories.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sample Interval (month)</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Left censored</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New IMI</td>
<td>1*</td>
<td>0</td>
</tr>
<tr>
<td>Total IMI in interval</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6X eligible</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Suspected IMI</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Suspected cure</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Sporadic IMI</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sporadic cure</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Right censored</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic IMI remaining at end of interval</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>number clinical events</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control Group</th>
<th>Sample Interval (month)</th>
<th>Total quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Left censored</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>New IMI</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total IMI in interval</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6X eligible</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Suspected IMI</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Suspected cure</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Sporadic IMI</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sporadic cure</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Right censored</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic IMI remaining at end of interval</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>number clinical events</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. Left censored, quarters entering pen with existing intramammary infection (IMI).
2. New IMI, quarters previously identified as negative in pen.
3. Eligible for extended therapy based on cow level SCC ≥ 100,000 cells/ml and current positive culture for *Streptococcus* species.
4. Treated once daily with intramammary injection of penicillin G (20,000 U) for eight days.
5. Paste tested not c cored as negative for presence of current type on 2 of 4 samples taken at 7 (± 2) day intervals.
6. Spontaneous cure defined as an IMI found negative for previous strain on of 2 samples taken at least 20 days apart.
7. Right censored, quarters exiting the pen with existing IMI.
8. *Number of asterisk indicates number of clinical events associated with either an existing IMI (flare-up) or a new IMI clinical at start.
Effect of treatment on duration of S. uberis and S. dysgalactiae mastitis

The mean (s.d.) and median duration of all S. uberis and S. dysgalactiae IMI are shown in table 4.5 and the frequency distribution of IMI duration categories demonstrates that the majority of IMI for both species had a duration of infection < 60 days (figure 4.2) which likely influences overall estimates of the effect of treatment on duration of infection for all infected quarters within a treatment group. Overall a numerical reduction in the duration of infection was observed for all S. uberis and S. dysgalactiae IMI in the treatment pens compared to control pens (table 4.5).

![Figure 4.2 Frequency distribution of Streptococcus dysgalactiae and Streptococcus uberis intramammary infections by duration of infection categories.](image-url)
In a generalized linear model treatment was not a significant predictor of LnIMIDUR when accounting for the affect of farm and the treatment by farm interaction, with least square means estimates of 22 and 36 days duration (back-transformed from the natural logarithm) for the treatment and control groups respectively.

Table 4.5 Duration of infection estimates for *Streptococcus uberis* and *Streptococcus dysgalactiae* intramammary infections. Values calculated from original (i.e. not logarithm transformed) data.

<table>
<thead>
<tr>
<th>Streptococcus uberis</th>
<th>all IMI</th>
<th>chronic IMI</th>
<th>Rx treated</th>
<th>not Rx treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated control</td>
<td>12</td>
<td>29</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>control</td>
<td>5</td>
<td>131</td>
<td>110</td>
<td>114</td>
</tr>
<tr>
<td>Farm 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated control</td>
<td>62</td>
<td>82</td>
<td>89</td>
<td>44</td>
</tr>
<tr>
<td>control</td>
<td>32</td>
<td>86</td>
<td>83</td>
<td>56</td>
</tr>
<tr>
<td>all</td>
<td>54</td>
<td>67</td>
<td>81</td>
<td>26</td>
</tr>
<tr>
<td>treated control</td>
<td>37</td>
<td>95</td>
<td>89</td>
<td>56</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Streptococcus dysgalactiae   |        |             |            |               |
| Farm 1                       |        |             |            |               |
| treated control              | 16     | 30          | 35         | 18            | 1             | 113          | 5           | 68           | 41           | 43           | 3           | 79           | 59           | 112          | 2           | 26           | 16           | 20           |
| control                      | 8      | 33          | 33         | 23            | 1             | 95           | 4           | 58           | 28           | 55           | -           | -            | -            | -            | 4           | 47           | 38           | 42           |
| Farm 2                       |        |             |            |               |
| treated control              | 3      | 35          | 27         | 35            | 6             | 60           | 2           | 49           | 15           | 49           | 1           | 38           | -            | -            | 1           | 60           | -            | 60           |
| control                      | 6      | 95          | 147        | 38            | 1             | 357          | 3           | 152          | 178          | 63           | -           | -            | -            | -            | 1           | 357          | -            | 357          |
| all                          | 19     | 31          | 33         | 19            | 1             | 113          | 7           | 62           | 35           | 43           | 4           | 88           | 43           | 75           | 3           | 49           | 16           | 38           |
| treated control              | 14     | 57          | 94         | 28            | 1             | 357          | 7           | 98           | 116          | 56           | -           | -            | -            | -            | 5           | 134          | 151          | 55           |

In a Kaplan-Meier estimate of the survival function of all IMI accounting for right censored observations, the median (95% CI; mean, s.d.) duration of infection was
estimated to be 38 (25-64; 77, 12) and 63 (55-129; 100, 14) days in the treatment and control groups respectively, but the overall survival curves were not different at the p≤0.05 level (log rank test chi-square= 2.81, p=0.094) (figure 4.3).

![Survival Distribution Function](image)

Figure 4.3 Kaplan-Meier survival function of *Streptococcus dysgalactiae* and *Streptococcus uberis* intramammary infections (IMI) from 2 commercial dairy herds accounting for right censored observations, including farm and bacterial species as covariates, and stratified by treatment groups (Treatment group = solid line; Control group = dashed line).

Effect of treatment on IMI prevalence and incidence

There were a total of 23 new *S. dysgalactiae* IMI per 500,653 quarter days at risk, with 17 new IMI observed on farm 1 and 6 new IMI on farm 2. There were 40 new *S.*
*uberis* IMI per 496,991 quarter days at risk, with 12 new IMI on farm 1 and 28 new IMI on farm 2. Figures 4.4 and 4.5 shows the relationship between prevalence and incidence for each pathogen stratified by farm and pen.

Figure 4.4 Incidence and Prevalence of *Streptococcus uberis* intramammary infections (IMI) stratified by farm and treatment group. Prevalence in quarter days infected is graphed on the right axis and represent by a line, while incidence of new IMI (quarters per 10,000 quarter days at risk) in naïve susceptible quarters (solid bars) and recovered susceptible quarters (open bars) are graphed on the left axis.
Figure 4.5 Incidence and Prevalence of *Streptococcus dysgalactiae* intramammary infections (IMI) stratified by farm and treatment group. Prevalence in quarter days infected is graphed on the right axis and represented by a line, while incidence of new IMI (quarters per 10,000 quarter days at risk) in naïve susceptible quarters (solid bars) and recovered susceptible quarters (open bars) are graphed on the left axis.

When data on *S. uberis* and *S. dysgalactiae* prevalence was pooled there was a strong effect of prevalence on incidence of new mastitis cases caused by these pathogens, which was also observed for *S. uberis* on farm 2, but not for any other subset of the data (i.e *S. dysgalactiae* on farms 1 and 2, and *S. uberis* on farm 1). Treatment was not a significant predictor of either prevalence or incidence when the models were applied to either the
complete data set, or subsets of data stratified by pathogen and farm. For incidence of \textit{S. uberis} on farm 2, time period was a significant predictor of incidence but the effects of treatment, or a treatment by time period interaction were not significant.

\textit{New IMI in recovered susceptible quarters following extended therapy}

On Farm 1, there was one new IMI due to \textit{S. uberis} identified in a quarter with a prior \textit{S. dysgalactiae} IMI that cured following extended therapy, and one quarter with a pre-treatment \textit{S. parauberis} IMI that had a post treatment IMI caused by \textit{L. lactis}. No Streptococcal IMI were observed in recovered susceptible quarters on farm 2.

Five IMI caused by other major mastitis pathogens were observed either during therapy or following the last day of treatment. On farm 2, four IMI caused by \textit{E. coli} (n=2) or \textit{Klebsiella} (n=2) were identified in 4 quarters of 3 cows during treatment by analysis of daily quarter milk samples taken prior to antimicrobial infusion on each of the 8 days of treatment. Positive sequential samples were observed for 2 or more days beginning on days 4, 6, 6, 7 for each of these 4 quarters respectively. Two of these quarters in one cow cured spontaneously by the first post treatment sample (pen move samples taken at 2 days post treatment), and one of these quarters in another cow was still positive for a \textit{Klebsiella} at 16 days following the last treatment before becoming negative on culture. Neither of these 2 cows showed clinical signs. In comparison one cow with \textit{E. coli} IMI that was first diagnosed on the sixth day of treatment was culled due to \textit{E. coli} mastitis within the week following treatment. On farm 1, a cow developed clinical
mastitis in two quarters at 16 days following extended therapy for streptococcal mastitis and a *Proteus* spp. was isolated from both quarters.

**Diversity of *S. uberis* strains**

Eight RAPD types were identified among 40 isolates examined from 21 quarters of 15 cows with *S. uberis* IMI on farm 1, and were arbitrarily numbered types M-1 through M-8. Of these, 7 different RAPD types were found to cause 10 IMI in 10 quarters of 9 cows, with each RAPD type only infecting up to 2 cows. In comparison RAPD type M-5 was associated with 11 IMI in 11 (52%) quarters of 7 (47%) cows on farm 1.

On farm 2, 30 RAPD types were identified among 104 isolates examined from 30 IMI in 27 quarters of 21 cows. Again, most RAPD types were isolated from only one or two quarters of one or two cows, but one RAPD type (S-9) was associated with 7 IMI in 7 (26%) quarters in 5 (24%) cows.

**Influence of speciation and strain typing on determination of IMI definition and bacteriologic cure**

There were two cases in two cows where GPCN cocci IMI were identified in pre- and post treatment samples, suggesting no cure following extended therapy, which were defined as cures because a different species was identified in the post treatment sample. These included a quarter with *S. dysgalactiae* pre-treatment that developed a new *S.*
 uberis IMI post treatment (figure 4.4), and a quarter with a pre-treatment S. parauberis IMI that had a post treatment IMI caused by L. lactis.

Strain typing of a series of S. uberis isolates collected from individual quarters over time allowed the IMI definitions to be refined for 5 quarters of 5 cows. In two cases of a series of isolates, RAPD typing found different strain types in sequential samples with < 1000 cfu/ml resulting in a reclassification of the IMI status for theses quarter to negative. In another series of isolates from a quarter with a chronic IMI, RAPD typing identified a different strain type at the earliest positive sample with 100 cfu/ml resulting in a reduction in the duration of infection estimate for that IMI. In one quarter, a series of 4 IMIs caused by different strains was identified. These separate IMI included an initial clinical event at 19 days in milk, followed by 3 separate subclinical IMI based on pairs of sequential samples each caused by a different strain over a 240 day period. Interestingly, this same cow had chronic IMI with recurrent clinical mastitis in two other quarters with each quarter IMI caused by a single but different strain type. The remaining cases were IMI that were redefined from a single quarter level IMI of long duration with ≥ 4 positive monthly samples, to 2 IMI of shorter duration caused by different strains in the same quarter at different time periods.

Association between antimicrobial susceptibility and bacteriologic cure

Antimicrobial susceptibility was examined for pairs of isolates taken at two separate time points (e.g. enrollment isolate and subclinical pre-treatment isolate) prior to extended therapy. For each testing method, there was agreement in susceptibility
classification within each pair of pre-treatment samples. Minor differences in either the
zone diameter (1-2 mm) or the MIC (1 dilution) were observed for most pairs of isolates
from the same IMI. In only one pair of *S. uberis* isolates did a difference in measurements
of a disc diffusion diameter for pirlimycin result in a different susceptibility classification
(12 mm for 1 isolate shown in figure 1 versus 20 mm for the other not included in figure
4.6). The smaller observed zone diameter was subsequently confirmed by repeated
testing of these two isolates. Four pairs of *S. uberis* pre-treatment isolates were classified
as pirlimycin resistant by MIC and susceptible by disc diffusion (figure 4.6). Thornsberry
et al. (1993) had previous defined falsely susceptible isolates (i.e. resistance by MIC and
susceptible by disc diffusion) as “very major errors”, and falsely resistant isolates (i.e.
susceptible by MIC and resistant by disc diffusion) as “major errors”. Using these
definitions we observed no major errors and 4 very major errors. None of these four *S.
uberis* IMI cured in the 4 weeks following therapy, although all appeared to
spontaneously cure after 30 days post treatment. Two out of 8 isolates classified as
pirlimycin resistant by MIC $\geq$ 4 ug/ml cured following extended therapy. These were
identified as *Enterococcus faecalis* and *Lactococcus lactis*. In comparison, 5 of the 6
isolates with an MIC $\geq$ 4 ug/ml that did not cure following extended therapy were *S.
uberis* representing a single RAPD type (M-5) from farm 1 and 2 RAPD types (S-1 and
S-9) from farm 2. The additional isolate with a pirlimycin resistant MIC that did not cure
was *Lactococcus lactis*. One out of 17 isolates classified as pirlimycin susceptible by
MIC $\leq$ 2 ug/ml did not cure following extended therapy. This isolate was a *S. uberis*
(RAPD type S-21) that demonstrated erythromycin resistance (MIC $\geq$ 8 ug/ml and no
zone of inhibition by disk diffusion) and was D-test positive for pirlimycin and clindamycin, but appeared sensitive to lincosamides in the absence of erythromycin induction.

Figure 4.6 Scatter plot for pirlimycin MICs and zone diameters observed for representative pre-treatment isolates of gram-positive catalase-negative cocci causing IMI that were treated with 8 days intramammary pirlimycin. Results of one isolate per IMI are shown. Open symbols = cure following 8 days therapy, Solid symbols = no cure following 8 days therapy, □ or ■ = Streptococcus uberis (n=17), ◇ or ◆ = Streptococcus dygalactiae (n=4), △ or ▲ = other ‘streptococci’ [Lactococcus lactis (n=2), Enterococcus faecalis (n=1), Streptococcus parauberis (n=1), Streptococcus pluranimalium (n=1), and Aerococcus spp. (n=1)]. Horizontal line represents the MIC breakpoint (≤ 2 ug/ml) is susceptible, and the vertical line represents the disk diffusion breakpoint (≥ 13 mm is susceptible) as recommended for gram-positive pathogens causing mastitis (CSLI, 2004). Isolates with footnotes: 1 = D-test positive S. uberis isolate, zone diameter in absence of Erythromycin induction = 21 mm, in presence of erythromycin induction = 10 mm; L= Lactococcus lactis; Ef= Enterococcus faecalis.
Therefore after reclassifying the *S. uberis* isolate with inducible pirlimycin resistance as resistant, in this study 11 of 11 pirlimycin susceptible *S. uberis* isolates cured, while 0 of 6 pirlimycin resistant *S. uberis* isolates cured. In contrast among the other streptococcal species, 5 of 5 susceptible isolates cured and 2 of 3 resistant isolates cured.

Pirlimycin resistance was identified in 6 *S. uberis* isolates from 4 different RAPD types, and was consistently associated with clindamycin resistance. In RAPD types M-5 and S-9, lincosamide resistance was associated with tetracycline resistance, while tetracycline resistance was not found in the remaining *S. uberis* isolates. *S. uberis* RAPD type 26 demonstrated intermediate resistance to ampicillin and penicillin, while the remaining *S. uberis* isolates appeared susceptible to all other antimicrobials tested (table 4.6).

**Table 4.6 Antimicrobial susceptibility profile of *Streptococcus* spp. isolates obtained pre-treatment.**

<table>
<thead>
<tr>
<th>Species</th>
<th>RAPD type</th>
<th>IMI</th>
<th>Amp</th>
<th>Fra</th>
<th>Pen/Nov</th>
<th>Cap</th>
<th>Xan</th>
<th>Ery</th>
<th>Fre</th>
<th>Clin</th>
<th>Tet</th>
<th>Ya</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. uberis</em></td>
<td>S-1</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-4</td>
<td>3</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>M-5</td>
<td>2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-7</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-9</td>
<td>2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-10</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-18</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-21</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-23</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-26</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-28</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>S-30</td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>not typed</td>
<td>4</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>E. dys-1</em></td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>E. parah</em></td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. parah</em></td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>A. spp</em></td>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

1. *S. uberis* = *Streptococcus uberis*, *S. dys-1* = *Streptococcus dysgalactiae*, *L. lactis* = *Lactococcus lactis*, *R. parah* = *Enterococcus faecalis*, *S. parah* = *Streptococcus parahominis*, *A. spp* = *Aerococcus spp*
2. Amp = ampicillin, Pen = penicillin, Nov = novobiocin, Cap = capreomycin, Xan = xanthin, Ery = erythromycin, Fre = fucidin, Clin = chloramphenicol, Tet = tetracycline, and Ya = vancomycin.
4. Erycine test positive, inducible resistance to pirlimycin and clindamycin.
Discussion

The direct and indirect effects of lactation therapy of subclinical mastitis due to *S. uberis* and *S. dysgalactiae* were not significant in the herds in this study. While the proportion of subclinically infected quarters that cured following intramammary treatment was in the range observed for *S. uberis* and *S. dysgalactiae* in previous studies (Gillespie et al., 2002; Deluyker et al., 2005; Sandgren et al., 2008) the spontaneous cure rate observed in this study was increased compared to other reports (Cattell et al., 2001; Gillespie et al., 2002; St Rose et al., 2003; Oliver et al., 2004; Salat et al., 2008; Sandgren et al., 2008). Differences in study design, specifically the longitudinal design of this study compared to other treatment trials that followed IMI for a shorter time period, likely contributed to our observation of a high spontaneous cure rate. Similar spontaneous cure proportions for the environmental streptococci have been observed in other longitudinal studies (Todhunter et al., 1995; Zadoks et al., 2003; McDougall et al., 2004) and in one treatment trial (McDougall, 1998).

To the best of our knowledge this is the first subclinical mastitis treatment trial that used duration of infection as an outcome measure. The durations of subclinical *S. uberis* infections observed in this trial did not differ from previous reports (Watt, 1999; Zadoks et al., 2003; McDougall et al., 2004). The finding of no difference between treatment and control groups in duration of subclinical IMI was likely influenced by the
large proportion of subclinical *S. uberis* and *S. dysgalactiae* IMI identified that spontaneously cured in less than 60 days. However, limiting analysis to only those quarters with IMI of greater than 30 days duration still resulted in no evidence of a treatment effect on duration of infection. It was clear that approximately 25% of *S. uberis* IMI have a duration of infection greater than 100 days and the majority of these are strictly subclinical in presentation, similar to what was reported previously (Zadoks et al., 2003). Targeting this group of infections for treatment would seem logical and would reduce overall duration of *S. uberis* infection in a population of dairy cattle. Further, treatment of the short duration infections is likely an imprudent use of antimicrobials and not cost effective (Hillerton and Berry, 2003). However, the challenge remains in distinguishing those infections that are destined to spontaneously cure within one to three months from those that are likely to survive for more than 90 days. It appears the enrollment criteria used in this study were insufficient in making that distinction. Further studies on the association between host or pathogen factors and duration of infection may provide prognostic markers that can be used in treatment decisions similar to has been suggested for *Staphylococcus aureus* infections (Barkema et al., 2006). Data from the current studies may be used to evaluate pathogen factors based on results of RAPD typing.

The use of antimicrobial susceptibility profiles to select therapies and infections for treatment remains controversial with differences of opinion among researchers (Constable and Morin, 2003; Barkema et al., 2006). Results of pre-treatment susceptibility testing of isolates indicated a strong association between MLS
susceptibility and probability of cure following extended pirlimycin therapy, with
treatment failures in 6 of 6 pirlimycin resistant *S. uberis* isolates and 0 of 11 susceptible
*S. uberis* isolates. Further work should be conducted to evaluate the potential value of
including susceptibility testing as a treatment criterion. These results can not be expanded
to other streptococcal species as response to therapy may not be completely associated
with susceptibility profiles but may be driven by other virulence factors in other species
(Haveri et al., 2007), thus explaining some of the conflicting results regarding use of
susceptibility profiles. The potential association between strain type and susceptibility
profile should also be considered in future studies as specific strains may differ in the
ability to acquire mobile genetic elements encoding for resistance (Waldron and Lindsay,
2006; Sung and Lindsay, 2007).

A further constraint in this study could have been the ability to only enroll a
limited number of cows for therapy within a given month. This may have allowed the
continued presence of infected individuals within the herd contributing to transmission.
Based on results obtained for herd 2 this potential limitation may be inconsequential in
many herds as we were able to eliminate the majority of *S. uberis* IMI over a 3 month
period.

The observation of potential iatrogenic IMIs association with extended
intramammary pirlimycin therapy appears to be consistent (Gillespie et al., 2002;
Middleton and Luby, 2008) and has led to the recent inclusion of a precautionary
statement on the product insert for the commercial product. Prevention of these infections
is critical to the successful implementation of lactation therapy and may be accomplished
to some degree by careful aseptic technique during intramammary infusion, or by the use of systemic therapy (Salat et al., 2008; Sandgren et al., 2008), although there are currently no systemic products approved for treatment of subclinical mastitis in the United States. A possible additional value of systemic therapy for subclinical mastitis may be that it provides simultaneous treatment of all four quarters and may eliminate minor mastitis pathogens infecting other quarters of a cow with a major pathogen in one or more quarters. However the effect of eliminating minor mastitis on the risk of new infections associated with major pathogens needs to be evaluated further (Lam et al., 1997a; White et al., 2006). The increased risk of new infections following elimination of minor infections may explain the observation that the risk of new mastitis treatments following treatment of subclinical mastitis did not differ among cows treated by an intramammary or systemic route (Sandgren et al., 2008).

While *S. uberis* is commonly described as an environmental pathogen, a few recent reports have provided evidence of contagious transmission of a dominant strain within some dairy cattle populations under specific management conditions (Phuektes et al., 2001b; Zadoks et al., 2001a). While these contagious outbreak scenarios may be infrequent among reports of mastitis epidemiology for the environmental streptococci, they do reinforce the concept that cow to cow transmission of mastitis pathogens is possible and contributes to the total infection pressure or force of infection experienced within a herd. Under most scenarios it appears the infection pressure associated with environmental sources of infection may exceed the pressure due to contagious sources for the ‘environmental’ streptococci including *S. uberis*. This appears to be the case for *S.*
uberis infections in herd 1 as incidence of new *S. uberis* IMI appeared to be unrelated to prevalence, despite finding approximately 50% of quarters infected with the same strain in this herd. An environmental source of exposure would be predicted based on the high degree of *S. uberis* strain diversity observed in herd 2, however this appears to conflict with the observation that incidence was associated with prevalence in this herd, and that in the treatment group elimination of infections over a 3 month period eliminated new *S. uberis* IMI in the subsequent months. It is unclear if other management factors may have played a role in this finding; however there were no observed changes in bedding management or environmental hygiene practices during the 13 months of the study on this farm.

Conclusion

This study found no significant direct or indirect effect of subclinical treatment targeting *S. uberis* and *S. dysgalactiae* mastitis although the proportion of infected quarters that cured following 8-day treatment was similar to that of other studies where a significant effect was observed. The observation that a large proportion of subclinical IMI that cured spontaneously during lactation in untreated cows contributed to the lack of a significant effect of treatment on the proportion of quarters that cured and the duration of infection. In one herd, incidence of new *S. uberis* infections was associated with prevalence and new *S. uberis* IMI were eliminated following successful treatment of existing *S. uberis* IMI in one group of cattle, suggesting contagious *S. uberis* transmission may have been a factor in this population. Antimicrobial susceptibility profiles
indicating erythromycin and pirlimycin resistance of pre-treatment S. uberis isolates was associated with treatment failure following extended pirlimycin therapy of subclinical mastitis.

Acknowledgements

The authors acknowledge the contribution of participating farm owners and farm staff. The support of our field and laboratory staff is also greatly appreciated. This project was supported in part by funding from Pfizer Animal Health, USDA Cooperative State Research, Education, and Extension Service (CSREES), National Research Initiative award # 2005-35204-15651, USDA-CSREES Vermont Agricultural Experiment Station Animal Health and Disease award # VT-AH01011, and the Multi-State Mastitis Research Project USDA CSREES NE-1028 (formerly NE-1009).

References


CSLI, 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility
Tests for Bacteria Isolated from Animals; Approved Standard. 3th ed. CLSI Wayne, PA.


Hogan, J.S., González, R.N., Harmon, R.J., Nickerson, S.C., Oliver, S.P., Pankey, J.W.,


St Rose, S.G., Swinkels, J.M., Kremer, W.D., Kruitwagen, C.L., Zadoks, R.N., 2003. Effect of penethamate hydriodide treatment on bacteriological cure, somatic cell count and milk production of cows and quarters with chronic subclinical...
Streptococcus uberis or Streptococcus dysgalactiae infection. J. Dairy Res. 70, 387-394.

Association between *Coxiella burnetii* shedding in milk and subclinical mastitis in dairy cattle

John Barlow¹, Brad Rauch²,³, Frank Welcome²,³, Sung Guk Kim³, Edward Dubovi³, and Ynte Schukken²,³

¹Department of Animal Science, University of Vermont, Terrill Hall 570 Main Street
Burlington, Vermont 05445

²Quality Milk Production Services, New York State College of Veterinary Medicine,
Cornell University, 22 Thornwood Drive Ithaca, New York 14850

³Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Abstract

The objective of this research was to explore the potential association between *Coxiella burnetii* shedding in milk and chronic subclinical mastitis in dairy cattle. In two separate studies, we identified an association between PCR-based detection of *C. burnetii* in milk and chronic subclinical mastitis in lactating dairy cows. These studies were conducted in a commercial dairy herd where there was ongoing intensive monitoring of subclinical mastitis by aerobic bacteriology, but no prior knowledge or management of *C. burnetii* infections. In a case-control study, quarter level *C. burnetii* status determined by real-time quantitative PCR (RT-qPCR) was strongly associated with chronic subclinical mastitis as measured by milk somatic cell counts. In a subsequent cross sectional study, 147 (45%) of 325 lactating cows were positive for *C. burnetii* by RT-qPCR of composite milk samples. In a generalized linear model, accounting for the effect of covariates including aerobic intramammary infection status, *C. burnetii* PCR status was a significant predictor of linear somatic cell count score. In agreement with a small number of previous reports, this research provides evidence that there may be mammary gland specific manifestations of *C. burnetii* infections in dairy cattle.
Introduction

*Coxiella burnetii* is an obligate intracellular zoonotic pathogen and the etiologic agent of Q fever in humans (Marrie and Raoult, 1997; Maurin and Raoult, 1999; McQuiston and Childs, 2002; Arricau-Bouvery and Rodolakis, 2005). Domestic ruminants (cattle, sheep and goats) are described as the primary reservoir species for exposure of humans (Marrie and Raoult, 1997; Maurin and Raoult, 1999; McQuiston and Childs, 2002; Arricau-Bouvery and Rodolakis, 2005). In dairy cattle *C. burnetii* infections may be under-recognized (McQuiston and Childs, 2002; McQuiston et al., 2006) and there is incomplete understanding of infection dynamics and disease manifestations. In the United States there are currently no approved *C. burnetii* veterinary diagnostic tests or vaccines, and no state or national coxiellosis surveillance or control programs for ruminant species, although recent publications have suggested that *C. burnetii* is enzootic in US dairy herds (McQuiston and Childs, 2002; Arricau-Bouvery and Rodolakis, 2005; Kim et al., 2005).

*C. burnetii* infections of domestic ruminants are generally described as subclinical (i.e. asymptomatic) and persistent (Marrie and Raoult, 1997; Maurin and Raoult, 1999; McQuiston and Childs, 2002; Arricau-Bouvery and Rodolakis, 2005). Where clinical signs have been reported, they are most frequently described in sheep and goats and are related to reproductive disease (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Arricau-Bouvery et al., 2005; Berri et al., 2005a; Berri et al., 2005b). Results of studies investigating the relationship between herd level seroprevalence and
reproductive health in dairy cattle are inconsistent (Literak and Kroupa, 1998; To et al., 1998; Bildfell et al., 2000). In past studies, a major limitation may have been the reliance on serology to define *C. burnetii* infection status, as serology is poor indicator of active *C. burnetii* shedding in individual animals (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Berri et al., 2005a; Berri et al., 2005b; Guatteo et al., 2006). Classically, confirmation of active infection in cattle required isolation of the organism either by laboratory animal or cell culture inoculation (Jellison et al., 1948; Bell et al., 1949; Biberstein et al., 1974; Lorenz et al., 1998; Arricau-Bouvery and Rodolakis, 2005), and currently few laboratories carry out isolation due to legal limitations, risk of human exposure and lack of sensitivity of the technique (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Guatteo et al., 2006). A small number of recent studies have described PCR-based DNA detection to identify shedding of *C. burnetii* in ruminants, including dairy cattle with reproductive disorders (To et al., 1998; Berri et al., 2005a; Berri et al., 2005b; Guatteo et al., 2006).

While *C. burnetii* is shed for extended periods in the milk of dairy cattle, and has been shown to be immunogenic in dairy cattle, potential associations with clinical or subclinical mastitis have only rarely been examined (Jellison et al., 1948; Bell et al., 1949; Biberstein et al., 1974; Marrie and Raoult, 1997; Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005). In reviews of mastitis etiologies, most authors recognize the potential public health significance of *C. burnetii* excretion in milk without describing the organism as a cause of mastitis (Heidrich and Renk, 1967; Rebhun, 1995). Some reviews have included *C. burnetii* among ‘the lesser known organisms that may
cause mastitis’ (Philpot and Pankey, 1975), while others did not include the organism among a broad review of bacterial etiologic agents of mastitis (Watts, 1988). In 1948 and 1949 respectively, a case of chronic focal mastitis in a cow naturally infected with \textit{C. burnetii} (Jellison et al., 1948), and severe acute mastitis following experimental intramammary inoculation of dairy cattle (Bell et al., 1949) were described. A more recent report suggested that the prevalence of \textit{C. burnetii} infections was higher among dairy cattle with reproductive problems including mastitis (To et al., 1998).

PCR has been described as the only veterinary diagnostic method to allow detection of specific \textit{C. burnetii} clinical manifestations such as metritis and as the ‘most sensitive and rapid means to identify shedders’ (Arricau-Bouvery and Rodolakis, 2005). PCR has been used to identify shedding patterns of \textit{C. burnetii} DNA in ruminant milk (Arricau-Bouvery and Rodolakis, 2005; Arricau-Bouvery et al., 2005; Berri et al., 2005b; Guatteo et al., 2006), although it is currently unclear whether shedding in milk is related to mammary specific manifestations of infection.

The objective of this work was to explore the potential association between \textit{C. burnetii} detection in milk by RT-qPCR and chronic subclinical mastitis in a commercial dairy herd undergoing intensive mastitis monitoring by standard methods.
Materials and methods

Herd description

This study was conducted on a commercial dairy herd in north-central New York, USA. The study herd consisted of approximately 350 lactating Holstein cattle housed in a free-stall confinement barn and milked in a double-6 parallel-stall parlor. Pregnant non-lactating cows were housed in two group pens, and small groups (≤ 4 cows) of periparturient cows were managed in a separate maternity pen with straw bedding cleaned regularly and replaced monthly. *C. burnetii* infection status of the herd was unknown prior to this study and there was no established surveillance or management targeting *C. burnetii* control. Sporadic and incidental metritis, infertility, and abortion events were recorded in the two years prior to this study, but no diagnostics of individual reproductive problems were completed, and no definitive causes of abortions were identified. The herd was demonstrated to be free of intramammary infections caused by *Streptococcus agalactiae* and *Mycoplasma* spp. based on extensive individual cow and bulk tank milk cultures over the proceeding 13 months.

*Coxiella burnetii* PCR

Prevalence of *C. burnetii* DNA sequence in milk was assayed by RT-qPCR of quarter or composite (all quarters within a cow) milk samples collected from individual cows using previously reported methods (Kim et al., 2005). Briefly, DNA from milk
was isolated using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Isolated DNA samples were tested using primers and a probe targeting the repetitive transposon-like region (1S1111a) of the C. burnetii genome (Hoover et al., 1992). Real-time PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Template DNA (5 µl) was added to a reaction mixture containing 0.3 µM each primer, 0.1 µM probe, 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 25 µl. The cycle profile of real-time PCR was as follows: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60s. A standard curve was constructed using a series of 10-fold dilutions of total genomic DNA (10^6 to 10^1 C. burnetii genome copies/ml) isolated from C. burnetii Nine Mile, phase II (RSA 493). DNase free water was used as the negative control (Hoover et al., 1992).

*Milk sampling, aerobic mastitis microbiology and somatic cell count analysis*

For three months prior to C. burnetii testing, monthly quarter milk samples were obtained aseptically for aerobic bacteriologic culture from all cows and held at 4 °C until processing for bacteriologic culture within 24 hours. In addition, quarter milk samples from all cows at calving, dry-off, and all cases of clinical mastitis, including pre- and post-treatment were collected and held frozen at -20 °C for a maximum of two weeks until processing for bacteriologic culture. Microbiological analysis of milk samples was conducted using established methods for identification of aerobic mastitis pathogens (Sears et al., 1993; Hogan et al., 1999). Composite milk samples were obtained for
determination of milk somatic cell count (SCC) by electronic counting within 3 days prior to monthly sampling for bacteriology.

**Definitions**

Aerobic bacteriologic status of quarters was defined based on culture results from milk taken at the time of sampling for *C. burnetii* PCR. For cows and quarters where at least 3 months serial culture results were available, aerobic intramammary infection (A-IMI) status was defined using definitions of Zadoks et al. (Zadoks et al., 2001a) with a slightly more restricted definition for uninfected quarters, which were defined as a quarters with no growth of a major mastitis pathogen on 3 of 3 consecutive samples, or no growth of a minor pathogen on at least 2 of 3 consecutive samples. Major mastitis pathogens included isolation of *Staphylococcus aureus*, *Streptococcus* spp., *Arcanobacterium pyogenes* or coliform species, while minor mastitis pathogens were defined as coagulase negative staphylococci, or *Corynebacterium* species. For analysis of the association between *C. burnetii* status and aerobic culture results, quarter level observations for aerobic culture were merged within cow to define a cow level aerobic bacteriologic or A-IMI status.

Subclinical mastitis status was defined as an elevated milk somatic cell count (SCC) ≥200,000 cells/ml for a single monthly observation, where no clinical event was observed in the 14 days preceding or following the SCC test date. Chronic subclinical mastitis was defined as elevated SCC for at least 2 consecutive months. All somatic cell
count measures were transformed to the industry standard logarithmic linear somatic cell count score (LSCS) for statistical analysis where $\text{LSCS} = \frac{\ln(\text{SCC}/100)}{0.693147} + 3$.

**Study design and statistical analysis**

Data were recorded in spreadsheet format (Microsoft Excel, Microsoft Corporation), and the data file was imported to a statistical software program for subsequent data management and analysis (SAS version 9.1, SAS Institute). Descriptive statistical analysis, including tests for normality, was completed for all variables (PROC UNIVARIATE or SAS/INSIGHT, SAS 9.1, SAS Institute Inc.). Two separate studies were conducted.

**Study 1**

A preliminary case-control study was conducted to explore the association between subclinical mastitis and *C. burnetii* shedding among A-IMI negative cows. *C. burnetii* RT-qPCR status of quarter milk samples was compared for 39 quarter milk samples from 10 cows with culture negative chronic subclinical mastitis (SCC $\geq 200,000$ cells/ml and no major pathogen isolated for 2 consecutive months; cases) and 39 quarter milk samples from 10 cows with culture negative continuous low SCC (SCC $< 200,000$ cells/ml and no major pathogen isolated for 2 consecutive months; controls). Case and control cows were matched on lactation number and days in milk. The association between the number of *Coxiella burnetii* RT-qPCR (CbPCR) positive quarters within cows and cow level subclinical mastitis status was examined using contingency tables.
and Cochran-Mantel-Haenszel statistics, with significance of association set at p<0.05. A
generalized linear model was used to test for differences in the mean average somatic cell
count score among cows stratified by number of CbPCR positive quarters, with
significance set at p<0.05.

Study 2

Data from a cross sectional study of all lactating cows in the herd was used to test
for an association between *C. burnetii* shedding in milk and subclinical mastitis in a
series of hierarchical linear and logistic regression models (PROC MIXED and PROC
GENMOD, SAS 9.1, SAS Institute Inc.) that included up to 7 individual predictors and
any 2-way interaction significant at the p≤0.05 level. The full linear regression model
using the 3 month average LSCS (AVLS) as the outcome variable was AVLS = β₀ + β₁
CbPCR + β₂ A-IMI + β₃ LACTGRP + β₄ DIMCAT + β₅ CLIN + β₆ MILK + β₇ ME305 + β₈ Xᵢ+βᵢ
Xᵢ + …., where CbPCR is *C. burnetii* infection status as measured by RT-qPCR, A-IMI is
the aerobic bacterial intramammary infection status, LACTGRP is the lactation group
number, DIMCAT is the days in milk category, MILK is the pounds of milk produced at
the most recent test, ME305 is the predicted 305 day mature equivalent milk production,
and CLIN is clinical mastitis status, of each individual cow. A-IMI status was a
categorical variable describing the pathogen specific infection status of the cow for
common species and groups of mastitis pathogens (i.e. *Staphylococcus aureus*,
*Streptococcus* spp., other major pathogens (OMP), coagulase negative Staphylococci
(CNS), other minor pathogens (OTHMINOR), and aerobic culture negative samples
(NEG)). Lactation group reflects the age of the cow and was a categorical variable with 3 categories defined as lactation 1 (primiparous cows), lactation 2 (cows in second lactation), and lactation 3 or greater. Days in milk is the number of days currently lactating, and 10 categories were defined by 30 day intervals from 1 day post partum to 270 days, with the final category defined as ≥ 270 days in milk. Clinical status was a binary categorical variable defined as 1 for cows that had a clinical mastitis event in the 14 days prior to or following milk sampling for RT-qPCR testing. Final model selection was based on goodness of fit tests including change in deviance observed for the two hierarchical models being compared, as well as comparison of Akaike’s information criteria (AIC) and Bayesian information criteria (BIC) fit statistics, with CbPCR forced into all models. The potential association between C. burnetii shedding in milk and subclinical mastitis was also evaluated using logistic regression models developed in the same manner, but where the dependent variable was a binary variable defining a cow as having a persistent high SCC for 3 of 3 sequential test dates prior to CbPCR testing. For all independent variables included in the final models, Pearson correlation coefficients and tests of multicollinearity were explored using the PROC CORR and PROC REG procedures, respectively (SAS 9.1, SAS Institute Inc.).
Results

*Bacteriologic culture and somatic cell counts*

Quarter milk samples were collected for aerobic bacteriologic culture from 351 cows. Three consecutive months of serial aerobic culture results were available to define A-IMI status for 222 cows, and one, two, and three months of SCC data were available for 195, 189, and 185 of these cows, respectively. At the cow level, 21 cows had IMI caused by a major pathogen, 84 cows had IMI caused by a minor pathogen, and 23 cows had quarters with IMI caused by two pathogen types (mixed IMI) being predominately *Streptococcus* spp. plus either *Corynebacterium* spp. or coagulase negative staphylococci.

*Study one - Case-control study results*

There were 31 CbPCR positive and 8 CbPCR negative quarters among cows with culture negative chronic subclinical mastitis, and 11 CbPCR positive and 28 CbPCR negative quarters among culture negative cows with persistent low SCC. Eight cows with chronic subclinical mastitis were CbPCR positive in all four quarters, and 2 cows with chronic subclinical mastitis were CbPCR negative in all four quarters. In comparison, 5, 3, and 2 cows with persistent low SCC had 0, 3, and 4 CbPCR positive quarters, respectively. The number of CbPCR positive quarters within cows was positively associated with chronic subclinical mastitis status of cows (Cochran-Mantel-Haenzel statistic p=0.0236, on two degrees of freedom). The mean LSCS (s.d) of the 10 cows with four CbPCR positive quarters was 4.82 (1.95), compared to means of 1.20 (0.56) and 2.72 (2.23) for cows with 1 and 0 positive quarters respectively.
Study two – cross sectional survey results

One-hundred-forty-seven (45%) of 325 cows tested were positive for *C. burnetii* and cows later in lactation at the time of testing were more likely to be found CbPCR positive (table 5.1).

*C. burnetii* PCR status was a significant predictor of AVLS in all linear models regardless of number of covariates included. All covariates except ME305 were significant predictors of AVLS in multivariate models, and no 2-way interactions were significant or found to improve model fit. Table 5.2 includes results of the final linear regression model indicating CbPCR was a significant predictor of somatic cell count after correcting for the effect of 5 significant covariates. The mean somatic cell count of CbPCR positive cows was numerically higher than CbPCR negative cows regardless of infection status, but the difference was greatest among A-IMI negative cows (figure 5.1). For example, among cows with an A-IMI caused by *S. aureus* the 3 month average LSCS of CbPCR positive and negative cows were 5.93 and 5.33, respectively. In comparison, the least squares mean 3 month average LSCS of A-IMI negative CbPCR positive cows was 4.25 compared to 2.51 for A-IMI negative CbPCR negative cows (figure 5.1). The median somatic cell counts calculated from the raw data for CbPCR positive and negative cows with an IMI caused by a major mastitis pathogen were 762,000 and 528,000 cells per ml, respectively, and for CbPCR positive and negative cows with an A-IMI caused by a minor mastitis pathogen were 233,000 and 203,000 cells per ml, respectively. In comparison, the median SCC of cows with no evidence of an intramammary infection caused by aerobic pathogens was 229,000 and 82,000 cells per ml, for CbPCR positive
Table 5.1 Number of *Coxiella burnetii* PCR (CbPCR) positive and negative cows stratified by aerobic pathogen intramammary infection (A-IMI) status, lactation group, and days in milk group

<table>
<thead>
<tr>
<th>Lactation group</th>
<th>Days in Milk Category</th>
<th>CbPCR Positive</th>
<th>CbPCR Negative</th>
<th>CbPCR Positive</th>
<th>CbPCR Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Major Pathogen IMI</td>
<td>Minor Pathogen IMI</td>
<td>No A-IMI</td>
<td>Major Pathogen IMI</td>
</tr>
<tr>
<td>1</td>
<td>1-90</td>
<td>5</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>91-180</td>
<td>12</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>181-270</td>
<td>11</td>
<td>12</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt; 270</td>
<td>36</td>
<td>17</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Sub total lact group 1</td>
<td>64</td>
<td>67</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>1-90</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>91-180</td>
<td>8</td>
<td>18</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>181-270</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>&gt; 270</td>
<td>11</td>
<td>13</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sub total lact group 2</td>
<td>30</td>
<td>47</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>1-90</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>91-180</td>
<td>9</td>
<td>13</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>181-270</td>
<td>19</td>
<td>21</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>&gt; 270</td>
<td>23</td>
<td>19</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Sub total lact group 3</td>
<td>53</td>
<td>64</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1-90</td>
<td>10</td>
<td>34</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>91-180</td>
<td>29</td>
<td>54</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>181-270</td>
<td>38</td>
<td>41</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2 Parameter estimates for the multivariate model of the association between QPCR status and the 3 month average linear somatic cell count score in 181 lactating dairy cattle.

**Model**  \( \text{AVLS} = \text{CbPCR} + \text{A-IMI} + \text{CLINICAL} + \text{DIMCAT} + \text{LACTGRP} + \text{MILK} \)

| Effect      | Estimate | Standard Error | DF  | T Value | Pr > |t| |
|-------------|----------|----------------|-----|---------|-------|---|
| Intercept   | 5.4244   | 0.4384         | 163 | 12.37   | <0.0001 |
| CbPCR       | 1.0460   | 0.2058         | 163 | 5.08    | <0.0001 |
| *S. aureus* | 2.1839   | 0.4156         | 163 | 5.25    | <0.0001 |
| *Strep spp.*| -0.2361  | 0.9608         | 163 | -0.25   | 0.8062 |
| OMP         | 2.1946   | 0.5352         | 163 | 4.10    | <0.0001 |
| CNS         | 0.6373   | 0.2572         | 163 | 2.48    | 0.0142 |
| Minor       | 0.1535   | 0.2565         | 163 | 0.60    | 0.5503 |
| Clinical    | 1.0912   | 0.3050         | 163 | 3.58    | 0.0005 |
| DIMCAT      | -        |                |     |         |       |
| LACTGRP     | -        |                |     |         |       |
| MILK        | -0.02174 | 0.0050         | 163 | -4.33   | <0.0001 |

and negative cows, respectively. From the final multivariate logistic model, the odds of a chronic high cell count among CbPCR positive cows are 3.92 (95% CI, 1.63 <OR<9.39) times that of CbPCR negative cows, after accounting for the effect of other variables. In both linear and logistic multivariate models, CbPCR status was also a significant predictor of either AVLS or chronic subclinical mastitis when aerobic bacteriologic culture status (based on a single milk culture at the time of PCR testing) was substituted for A-IMI status (data not shown). Significant correlations were found between CbPCR status and DIMCAT (correlation coefficient = 0.255, p<0.001), MILK and DIMCAT (-0.393, p<0.001), CLIN and MILK (-0.145, p=0.009), and A-IMI status and DIMCAT (0.138, p=0.049); all remaining correlation coefficients were < 0.08. Further, no problem
of collinearity was identified as all variance inflation factor values were < 1.5, and the variance inflation factor for CbPCR and A-IMI status was 1.04 and 1.02, respectively.

Figure 5.1 Least square mean 3 month average linear somatic cell count score for CbPCR positive and negative cows stratified by aerobic intramammary infection (A-IMI) status where A-IMI status was based on at least 3 monthly serial aerobic bacteriologic culture results (error bars indicate standard error, number above bars = number of cows, A-IMI categories defined in text). * Among cows that were defined as A-IMI negative (n=73) the mean 3 month average LSCS of CbPCR positive cows was significantly greater (\(p=0.002\)) then that of CbPCR negative cows.
Discussion

In comparison to previous studies suggesting \textit{C. burnetii} is associated with bovine mastitis, a major strength of our studies is that they were conducted in a herd where there was ongoing intensive monitoring of subclinical mastitis infection dynamics. A key finding was that \textit{C. burnetii} shedding in milk was associated with chronic subclinical mastitis in cows demonstrated to be free of infection caused by common aerobic mastitis pathogens. This is in contrast to three previous reports of mastitis associated with \textit{C. burnetii} infection that did not appear to investigate the possibility of mastitis caused by common aerobic mammary pathogens (Jellison et al., 1948; Bell et al., 1949; To et al., 1998). Although we were able to account for the potential effect of bacterial mastitis pathogens identified by routine aerobic culture, we could not eliminate the possible role of other less common bacterial or viral pathogens that might be associated with subclinical mastitis (Philpot and Pankey, 1975; Watts, 1988; Wellenberg et al., 2002). Despite this limitation, our finding that A-IMI negative CbPCR positive cows had a median SCC of > 200,000 cells/ml compared to <100,000 cells/ml for A-IMI negative CbPCR negative cows is of particular interest from the perspective of mastitis control, as SCC is used as a diagnostic screening test in mastitis control programs (Sears et al., 1993). Further, the observation that the SCC of cows with both \textit{C. burnetii} positive milk and A-IMI was increased relative to CbPCR negative A-IMI positive cows, suggests a possible effect of co-infection of \textit{C. burnetii} and common mastitis pathogens, although the nature and clinical relevance of this potential interaction is unknown. Other
associations observed in this study that warrant further analysis include the finding that CbPCR positive status increased with increasing days in milk. Because the history and true prevalence of *C. burnetii* infections in this herd was unknown it is unclear how our observations may have been affected by duration of *C. burnetii* infection in the herd or individual animals. Further, because the organism was not isolated and the strain type was not identified it is unknown if the mammary specific disease manifestations observed in this herd could have been influenced by the involved strain(s) (Arricau-Bouvery et al., 2006).

These studies were limited to PCR-based detection of *C. burnetii* DNA sequence in milk samples, so we are unable to speculate on the viability of organisms in milk of cows in this commercial herd, or on the sensitivity and specificity of the PCR relative to other diagnostic methods. Current alternatives to diagnosis of *C. burnetii* infection in ruminants include serological analysis, organism isolation by either cell culture (e.g. shell vial culture) or live animal inoculation, immunohistochemical and PCR-based detection (Marrie and Raoult, 1997; Maurin and Raoult, 1999; Bildfell et al., 2000; Arricau-Bouvery and Rodolakis, 2005). Previous reports have demonstrated that viable organisms may be variably shed in the milk of dairy cattle for extended periods of greater then 1 year and across multiple lactations (Biberstein et al., 1974; Marrie and Raoult, 1997; Arricau-Bouvery and Rodolakis, 2005), and that within a herd the proportion of cows shedding in milk may range from 15 to 62% (Biberstein et al., 1974; Guatteo et al., 2006). Our finding that 45% of cows in this commercial herd were CbPCR positive in milk samples is consistent with these previous reports. To the best of our knowledge,
only two studies have compared results of PCR detection of *C. burnetii* in milk with bacteria viability assays by mouse inoculation (To et al., 1998; Arricau-Bouvery et al., 2005). While these two studies demonstrate PCR positive milk samples contain viable organisms, additional studies are needed to determine how PCR based detection relates to the potential infectiousness of *C. burnetii* in milk samples, or the sensitivity and specificity of PCR relative to inoculation or antigen detection assays. The ability to obtain such data is likely restricted to a relatively small number of research laboratories, as isolation must be conducted in biosafety-level 3 laboratories (Arricau-Bouvery and Rodolakis, 2005), and may be legally restricted in the United States where the organism is classified as a category B bioterrorism agent by Centers for Disease Control and Prevention (CDC).

*Coxiella burnetii* infections of dairy cattle have received relatively little attention in North America (Bildfell et al., 2000). Shedding of the organism from other tissue sites and other potential disease manifestations were not explored in this herd. While our studies were limited to a single commercial dairy herd and focused on mammary specific detection of *C. burnetii* to evaluate a potential association with subclinical mastitis, the finding that *C. burnetii* PCR status in milk is associated with increased SCC, an economically important milk quality measure and diagnostic tool for mastitis control, suggests that additional research is justified to evaluate the consequences of *C. burnetii* infections in dairy cattle.
Acknowledgements

The authors would like to acknowledge the contribution of the farm owner and farm staff and his veterinary practitioner in conducting this study. The support of our field and laboratory staff is also greatly appreciated.

References


Philpot W. N. and Pankey J. W., Review of microorganisms that reportedly cause mastitis, Hill Farm Research Station, Homer, LA, 1975, pp. 118-120.


General Discussion and Summary

Discussion

The objective of this research was to evaluate the effects of lactation antibiotic therapy for the treatment of subclinical mastitis caused by the major gram-positive mastitis pathogens *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae*. This was addressed in a three-tiered approach that integrated a mathematical epidemiologic model, a randomly controlled clinical field trial, and molecular and microbiologic epidemiologic diagnostics.

Through the development of a deterministic model of mastitis transmission dynamics it was predicted that lactation therapy of subclinical mastitis could have strong positive herd level effects by reducing the size of the infected pool and thus indirectly affecting the risk of new infections for contagious pathogens. These findings are consistent with established concepts of infectious disease transmission but are unique in that they demonstrate the effect of a targeted antibiotic based intervention applied at the level of the individual for mastitis control in a population. Only a handful of studies have previously used predictive models to evaluate the impact of mastitis control interventions (Lam et al., 1996; Allore and Erb, 1999; White et al., 2001a; White et al., 2001b; White et al., 2002; Zadoks et al., 2002a; White et al., 2006), and none have modeled the impact
of a targeted intervention. Similarly, in human infectious disease predictive models most interventions evaluated are applied broadly across a population (Anderson and May, 1991; Halloran et al., 1991; Bonhoeffer et al., 1997; Pourbohloul et al., 2003).

Perhaps the most interesting finding from the simulations was the prediction that under high transmission scenarios the indirect effect of therapy becomes negative and the overall effect of therapy approaches zero. In this model structure, successful treatment of an infected individual resulted in a state shift to the recovered susceptible state which, under high transmission scenarios quickly returns to the infected state. For many diseases where there is strong host immunity this would seem paradoxical. However because there seems to be little protective effect of humoral immunity on risk of new mastitis infections for many pathogens including S. aureus and S. uberis, other host factors may govern the risk of infection. Therefore it is conceivable that a host that has been cured might be at increased susceptibility compared to previously uninfected animals that have been under the same level of exposure. For example, differences in host innate immunity may manifest as an a priori increased susceptibility which increases risk of infection for the life of that individual (Burvenich et al., 2004; Rainard and Riollet, 2006). Also, physical differences in teat end shape or sphincter length may lead to heterogeneity of infection risk within a population (Lacy-Hulbert and Hillerton, 1995). The assumption that drives the prediction of increased rates of infection from the recovered susceptible state is based on prior research demonstrating increased risk of new infections for quarters in the recovered susceptible state compared to the naïve susceptible state (Zadoks et al., 2002a). These results were further supported by observations of
reinfections during the field trial described in chapter 3. There are two practical implications that arise from these predictions. First, producers and their advisors would be wise to address factors that affect mastitis transmission before adopting lactation therapy as a component of mastitis control. This is consistent with early recommendations (Dodd et al., 1969; Dodd and Neave, 1970; Kingwill et al., 1970; Dodd et al., 1977) and recent predictive models (White et al., 2006). Second this highlights the potential value of culling as an important component of mastitis control (Dodd et al., 1977).

Our research suggests that prior economic models might be reformulated to incorporate the apparent non-linear relationship between transmission rates and the indirect effect of lactation therapy. In economic models Swinkels et al. (Swinkels et al., 2005a; Swinkels et al., 2005b) estimated the indirect effect of treatment as a constant function across two transmission scenarios. Data from our current study would suggest the proportion of prevented infections associated with treatment is not constant, but a complex function of cure rates, transmission rates, and the difference in the force of infection for naïve susceptible and recovered susceptible quarters. Our results suggest that there may be a range of transmission rates where the indirect effect of lactation therapy of subclinical mastitis may be positive; however in high transmission scenarios the indirect benefit of treating chronic subclinical mastitis may be limited, and the economic assumptions underlying previous models may not be constant. Additional research is needed to integrate our results into an economic model.
Another consideration to include in the economic models of lactation therapy should be the pathogen specific impact of the diagnostic component. Additional research is needed to evaluate the diagnostic component of targeted treatment for subclinical mastitis. The hypothesis would be that *S. aureus* treatment based on serial cow level SCC results and a single bacteriologic culture would result in few false negative or inappropriate treatment delays (high sensitivity for identifying a true *S. aureus* IMI) and few false positive inappropriate treatments (high specificity for identifying true negative *S. aureus* IMI). The risk of delayed treatment may be a reduced probability of cure, although false negative culture results in true *S. aureus* IMI appear to be infrequent enough that treatment would not be delayed for more than 1 to 2 months under a monthly surveillance system (Erskine and Eberhart, 1988; Sears et al., 1990). The cost of these false negatives would be an increase in the number of cultures needed prior to initiating treatment. The costs of false positive *S. aureus* diagnoses may be greater as this would lead to an inappropriate use of antimicrobials and an inappropriate increase in amount of discarded milk. The data from a series of previous field trials, including the one in this research, could be pooled to evaluate the diagnostic criteria used in this study for identification of specific pathogens causing subclinical mastitis. Studies similar to this have been conducted for evaluation of the California mastitis test as a component of a mastitis treatment program (Wallace et al., 2004). This may be especially important as managers of herds with bulk tank somatic cell counts in a moderately high to high range (e.g. > 250,000 cells/ml) may be considering lactation therapy as an adjunct to their current control practices and additional information on the use of sequential monthly
SCC results and bacteriologic culture is needed to implement such programs (Middleton et al., 2004).

A major limitation identified in this research and previously reported (Gillespie et al., 2002; Middleton and Luby, 2008) was the occurrence of new clinical or subclinical IMI due to coliform organisms following extended therapy. This has been previously attributed to a number of non-host associated factors including introduction of pathogens during intramammary therapy, use of narrow-spectrum antimicrobials, poor environmental hygiene, and seasonal effects of increased heat and humidity. Regardless of the cause, these infections severely impacted the response to therapy including the potential for a post-treatment reduction of SCC, and in a few cases resulted in the culling of a quarter or cow following treatment. The risk of these post-treatment infections should be included in future economic models unless they can be prevented. Strict attention to aseptic methods during infusion might significantly reduce the risk of these infections, however it is not clear that this is the only contributing factor. Systemic therapy might be considered as an alternative to intramammary infusion and would eliminate either the possibility of iatrogenic cases due to contamination of infusion products or the constant manipulation and potential irritation of the teat end and canal. However, Sangren et al. (2008) reported the same number of new mastitis treatments following systemic or intramammary treatment of subclinical mastitis suggesting host or environmental factors may also affect the risk of post-treatment infections.

In this research host factors associated with bacteriologic cure following treatment of subclinical mastitis were not addressed but might be used to select cows for treatment
as has been previously suggested (Sol et al., 1997; Deluyker et al., 2005; Barkema et al., 2006). However, at a population level this would suggest that some infected cows would be left in the herd unless they are removed or segregated based on culture status.

Selective lactation therapy is an alternative strategy that would need to be tested in epidemiologic and economic models as the indirect effect is likely to be reduced and may not off-set the reduced income associated with lost milk.

Pathogen factors associated with cure were examined in this research although they were limited to antimicrobial susceptibility testing. Antimicrobial resistance appeared to be associated with strain type in the herds in this study for both *S. aureus* and *S. uberis*. An interesting finding was the difference in response to treatment seen for the resistant strains of the two species. For *S. uberis* treatment failures were observed for 100% (n=6) of the isolates expressing resistance in vitro, and resistance was restricted to 5 of the 12 RAPD types tested. In comparison, among *S. aureus* isolates, treatment success was observed for 89% (8/9) of isolates carrying beta-lactam resistance. In these isolates the phenotype was limited to penicillin and ampicillin resistance out of 8 classes of antimicrobials examined. The lack of lincosamide and macrolide resistance in these isolates may explain treatment success, however it has been reported that penicillin resistance is a predictor for treatment failure to other classes of antimicrobials (Barkema et al., 2006). For either pathogen, in vitro resistance may be associated with other virulence factors that limit probability of cure (Haveri et al., 2005; Dingwell et al., 2006). It is hypothesized that there is an association between strain type and carriage of resistance among bovine mastitis pathogens. In the context of the broader hypothesis of
host-adapted and non-host-adapted strains of mastitis pathogens, a positive association between non-host-adapted strains and antimicrobial resistance gene carriage in *S. aureus* would explain observations of limited changes in the proportion of susceptible *S. aureus* mastitis isolates over the past decades (Erskine et al., 2002; Makovec and Ruegg, 2003). An association between strain-type and antimicrobial resistance may also explain the limited extent of differences in antimicrobial susceptibility of *S. aureus* on organic and conventional dairy farms (Tikofsky et al., 2003; Sato et al., 2004). The resistant phenotypes and genotypes of isolates obtained in this study were restricted to 2 of 11 PFGE types, consistent with other reports describing an association between PFGE type and susceptibility profiles (Anderson et al., 2006). Further analysis of the isolates from this study indicate that the resistant isolates are limited to multilocus sequence types (MLST) 8, 25, and 87 (table 6.1). ST 8 and 25 were found only on farm 2 and were associated with intramammary infections. MLST type 8 caused 8 IMI in 8 quarters of 6 cows, all of which cured following extended pirlimycin therapy. MLST type 25 was found in 1 quarter of 1 cow which did not cure immediately following therapy, but had cured by day 21 post-treatment. On farm 1 a single isolate of MLST type 87 was obtained from a milk sample of a cow with no evidence of mastitis and was classified as an incidental isolation presumed to be a contaminant. This isolate expressed methicillin resistance and was positive on a meca gene PCR. These five strain types are unique among MLST types that have been isolated from dairy cattle and are not associated with bovine *S. aureus* clonal complexes but cluster with human clonal complexes.
Table 6.1 Frequency of *Staphylococcus aureus* strain types isolated from 2 dairy herds including pulsed-field gel electrophoresis (PFGE) type and multilocus strain type (MLST) groupings.

<table>
<thead>
<tr>
<th>Strain type</th>
<th>PFGE(^1)</th>
<th>MLST(^2)</th>
<th>MLST CC(^3)</th>
<th>Herd 1 Isolates(^4)</th>
<th>cows</th>
<th>quarters</th>
<th>Herd 2 Isolates(^4)</th>
<th>cows</th>
<th>quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>151</td>
<td>151</td>
<td>35 (7)</td>
<td>21</td>
<td>24</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1a</td>
<td>New siv151</td>
<td>1</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1b</td>
<td>New siv151</td>
<td>20 (5)</td>
<td>29 (2)</td>
<td>11</td>
<td>13</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1c</td>
<td>nd</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>New siv352</td>
<td>97</td>
<td>9</td>
<td>3</td>
<td>16</td>
<td>13</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>nd</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>nd</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3 (^R)</td>
<td>8</td>
<td>8</td>
<td>16 (13)</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>352</td>
<td>97</td>
<td>3</td>
<td>2 (2)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>352</td>
<td>6 (3)</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4b(^n)</td>
<td>nd</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>97</td>
<td>97</td>
<td>3 (1)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5a</td>
<td>nd</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>nd</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7(^t)</td>
<td>20</td>
<td>20</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>479</td>
<td>479</td>
<td>4 (3)</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9(^R)</td>
<td>87</td>
<td>1</td>
<td>1 (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10(^R)</td>
<td>25</td>
<td>25</td>
<td>3 (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>11(^t)</td>
<td>nd</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

1 PFGE types with different numbers are major variants, with the same number and different letters are minor variants
2 MLST type, siv=single locus variant not currently submitted to database for type assignment, div=double locus variant not currently submitted to database for type assignment
3 MLST Clonal complexes predicted founder strain determined by eBURST MLST database (last searched January, 2008)
4 number isolates typed by MLST shown in parentheses
\(^t\) type found as only incidental isolate, not defined as IMI or associated with subclinical mastitis (cow SCC<200,000 cells/ml)
\(^R\) - fresh heifer clinical isolate did not enter study pens
\(^n\) - 100% of isolates were β-lactamase resistant phenotype and genotype
\(\text{nd} \) - not determined

Further research appears justified to test the hypothesis that antimicrobial resistant *S. aureus* isolates associated with mastitis belong to human associated clonal complexes (non-host-adapted) while bovine mastitis isolates associated with host-adapted clonal
complexes have rarely acquired antimicrobial resistance. These differences might be explained by differences in the ability of specific strains to acquire mobile genetic elements encoding resistance (Waldron and Lindsay, 2006; Sung and Lindsay, 2007), or may be related to the concept that there is no selective advantage for the acquisition of antimicrobial resistance genes among bovine host-adapted mastitis ecotypes.

In potential contrast, a unique finding of these studies was the unexpected high frequency of macrolide-lincosamide resistance among \textit{S. uberis} isolates. At the time these studies were initiated there was limited evidence of either erythromycin or pirlimycin resistance among streptococcal mastitis isolates, but in recent years an increase in the proportion of GPCN cocci expressing antimicrobial resistance has been observed (Erskine et al., 2002; Rossitto et al., 2002; Makovec and Ruegg, 2003; Loch et al., 2005; Schmitt-Van de Leemput and Zadoks, 2007). To the best of our knowledge this is the first study that has shown a clear relationship between antimicrobial susceptibility to pirlimycin and treatment outcome for subclinical \textit{S. uberis} mastitis. Previous studies that have demonstrated no predictive relationship between antimicrobial susceptibility and treatment outcome for \textit{Streptococcus} spp. have been limited to treatment of clinical mastitis and have either pooled results across all GPCN species (Hoe and Ruegg, 2005) or had included a limited number of resistant isolates (Constable and Morin, 2002). Further, we have identified a relationship between RAPD type and resistance phenotype. Additional research is justified to explore the potential associations between \textit{S. uberis} strain type, carriage of antimicrobial resistance genes, and response to treatment. Further, data from these studies and that of Schmitt-Van de
leemput and Zadoks (2007) suggest that the disc diffusion breakpoints for pirlimycin treatment of *S. uberis* mastitis should be evaluated.

Summary

This research provides data that may be used to inform decisions on the adoption of lactation therapy as a component of a mastitis control program. While there may be a number of individual herds that can successfully implement use of lactation therapy as a part of their mastitis control program, under current dairy herd management and economic conditions in North America it seems unwarranted to broadly recommend blanket adoption of this practice.


Kingwill, R.G., Neave, F.K., Dodd, F.H., Griffin, T.K., Westgarth, D.R., Wilson, C.D., 1970. The effect of a mastitis control system on levels of subclinical and clinical mastitis in two years. The Veterinary Record 87, 94-100.


