Towards a Better Understanding of the Epidemiology of Naturally Occurring
Staphylococcus aureus Intramammary Infections

Dissertation

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**ABSTRACT**

*Staphylococcus aureus* (SA) is the most prevalent contagious mastitis pathogen on US dairies and contributes significantly to cow morbidity and mortality, and loss of revenue. Despite its prevalence, our understanding of the epidemiology of naturally occurring SA intramammary infections (IMI) is limited. The research described herein attempts to provide a basic understanding of the epidemiology of naturally occurring SA IMI.

Assuming a disease free status in quarters initially culture negative for SA and a disease positive status in quarters that were culture positive with ≥1cfu/0.01mL within the first 3 samples, the sensitivity and specificity of microbiologic culture was evaluated using 2 different inoculum volumes. Using samples collected during two studies examining shedding patterns of SA, we found that the sensitivity of microbiologic culture for detecting SA IMI using a 0.01mL inoculum was 91% and the specificity was 99.4%. Using a larger (0.1mL) inoculum the sensitivity increased to 96.8% and the specificity was 99.3%. This study demonstrated a clear benefit of using a larger inoculum volume (0.1mL) for culture in the microbiologic detection of SA IMI.

The first study evaluated daily shedding patterns of SA over three 21 day sample periods. It was demonstrated that the variability in shedding had little consequence on our ability to accurately detect a SA IMI. Although the amount of SA recovered from infected quarters varied tremendously, 97.7% of the samples were culture positive. There were no consistent patterns of shedding identified or a relationship between shedding of SA and SCC. Using pulse field gel electrophoresis (PFGE) to evaluate the association of strain type and shedding of SA, quarters...
grouped into PFGE pulsortype 1 shed at consistently higher levels and with comparatively little variability, (median cfu/0.01mL=100) while quarters placed in PFGE pulsortype 2 shed SA at consistently lower levels (median cfu/0.01mL=59, p< 0.0001).

The second study examined naturally occurring SA IMI on 2 different dairies over an entire lactation further demonstrating a consistent recovery of SA from naturally infected quarters provided a large enough (0.1mL) inoculum was used. The sensitivity of culture was 95%, compared to 85% when using a smaller volume (0.01mL). Again, there were no consistent shedding patterns, other than that of consistently higher versus consistently lower. Quarters shedding consistently higher levels of SA were again grouped into PFGE pulsortype 1 further supporting the influence of strain type on either persistence or pathogenicity. Strains clustered into the PFGE type 1 had a median cfu/0.01mL of 83 while strains grouped into PFGE type 2 had a median cfu/0.01mL of 17.

The third study examined the effect of strain type on SA IMI cure rates. Evaluating cure by parity, 42% of the first lactation cows cured while only 10% of the multiparous cows cured. Unlike other published works, we found no significant difference (p=0.64) in cure rates in quarters with low quarter level SCC (200,000 cells/mL) and high SCC. Nor was there any significant difference (p = 0.35) when evaluating cure using composite test day information.

In conclusion, when using a larger inoculum volume, the influence of varied levels of bacterial shedding on the microbiological diagnosis of SA IMI is minimal providing a cost effective and efficient means by which to identify quarters with SA IMI. While there were no distinct shedding patterns or cycles identified in either bacterial or somatic cell count shedding, quarters infected with bacteria grouped into pulsortype 1 shed at significantly higher levels and experience fewer cures over the dry period. This finding highlights the potential application of pre-screening heifers prior to parturition for SA IMI and possible dry cow therapy providing the opportunities to cure a SA IMI before the heifer enters the milking parlor.
The worst sin towards our fellow creatures is not to hate them, but to be indifferent to them. That's the essence of inhumanity.

George Bernard Shaw

This one’s for my ladies, you deserve better.
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# Tables of Contents

**Abstract** .................................................................................................................. ii  
**DEDICATION** ........................................................................................................ iv  
**ACKNOWLEDGMENTS** .......................................................................................... v  
**VITA** ...................................................................................................................... viii  
**LIST OF TABLES** .................................................................................................... xii  
**LIST OF FIGURES** ................................................................................................... xiii

## Chapters:

1. **INTRODUCTION** ........................................................................................................ 1  

2. **Impediments to Understanding the Epidemiology of Bovine *Staphylococcus aureus* Intramammary Infections** ....................................................................................... 17  

   2.1 Our Current Understanding of the Epidemiology of *S. aureus* Mastitis .......... 17  
      2.1.1 The Prevalence of *S. aureus* mastitis ..................................................... 17  
      2.1.2 Sources and Transmission of *S. aureus* .................................................. 18  
      2.1.3 Prevention of *S. aureus* .......................................................................... 21  

   2.2 Diagnosis *S. aureus* Intramammary Infections ................................................ 22  
      2.2.1 Defining Intramammary Infection .............................................................. 23  
      2.2.2 Microbiological Diagnosis of *S. aureus* Intramammary Infections .......... 24  
      2.2.3 Defining Chronicity ................................................................................... 32  
      2.2.4 Diagnosing *S. aureus* using Somatic Cell counts .................................... 33  

   2.3 Treatment of *S. aureus* ...................................................................................... 36  
      2.3.1 Defining Cure ............................................................................................ 38  
      2.3.2 Treatment and the Utility of antibiotic Profiling ....................................... 40  
      2.3.3 Stage of Lactation and its Impact on Treatment Success ......................... 44  

   2.4 Strain Differences in *S. aureus* .......................................................................... 47
2.4.1 Interpretation of PFGE ................................................................. 48
2.4.2 Strain Diversity of S. aureus ...................................................... 49

2.5 Motivation for Research ............................................................... 54

2.6 Endnotes .................................................................................... 56

2.7 References .................................................................................. 57

3. THE EFFECTS OF INOCULUM VOLUME ON THE MICROBIOLOGICAL DETECTION OF NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS .............................................................. 66

3.1 Introduction ................................................................................ 66

3.2 Material and Methods ................................................................. 67

3.3 Results ....................................................................................... 69
  3.3.1 Using a 0.01mL Inoculum Volume ........................................... 69
  3.3.2 Using a 0.1mL Inoculum Volume ............................................ 69

3.4 Discussion .................................................................................. 70

3.5 References .................................................................................. 75

4. VARIATION IN DAILY SHEDDING PATTERNS OF STAPHYLOCOCCUS AUREUS AND SOMATIC CELL COUNTS IN NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS .......................................................... 78

4.1 Introduction ................................................................................ 78

4.2 Materials and Methods ............................................................... 79
  4.2.1 Study Population and Sampling .............................................. 80
  4.2.2 Strain Comparisons ............................................................... 80
  4.2.3 Statistical Analysis ............................................................... 83

4.3 Results ....................................................................................... 84
  4.3.1 Bacterial Shedding Patterns ................................................... 85
  4.3.2 Somatic Cell Counts ............................................................. 86
  4.3.3 Strain Differences ............................................................... 87

4.4 Discussion .................................................................................. 88
4.5 References........................................................................................................................................94

5. SHEDDING PATTERNS OF NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS
INTRAMAMMARY INFECTIONS SAMPLICD WEEKLY THROUGHOUT LACTATION............107

5.1 Introduction......................................................................................................................................107

5.2 Materials and Methods..................................................................................................................108
  5.2.1 Study Population and Sampling.................................................................................................108
  5.2.2 Strain Comparisons....................................................................................................................110
  5.2.3 Statistical Analysis......................................................................................................................111

5.3 Results...............................................................................................................................................113
  5.3.1 Bacterial Shedding Patterns.......................................................................................................114
  5.3.2 Somatic Cell Counts....................................................................................................................115
  5.3.3 Strain Differences.......................................................................................................................116

5.4 Discussion........................................................................................................................................119

5.5 References.......................................................................................................................................126

6. THE EFFECT OF STRAIN TYPE ON CURE-RATES IN DAIRY COWS WITH NATURALLY
OCCURRING STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS..............136

6.1 Introduction......................................................................................................................................136

6.2 Materials and Methods..................................................................................................................137
  6.2.1 Study Population and Sampling.................................................................................................137
  6.2.2 Strain Comparisons....................................................................................................................138
  6.2.4 Antibiotic Profiling.....................................................................................................................139
  6.2.5 Statistical Analysis......................................................................................................................139

6.3 Results...............................................................................................................................................140

6.4 Discussion........................................................................................................................................141

6.5 References.......................................................................................................................................144

7. DISCUSSION.....................................................................................................................................148

7.1 Major Findings of Current research...............................................................................................148
7.2 The Effects of Inoculum Volume on the Microbiological Detection of Naturally Occurring Staphylococcus aureus Intramammary Infections..........................149

7.3 Variation in Daily Shedding Patterns of Staphylococcus aureus and Somatic Cell Counts in Naturally Occurring Staphylococcus aureus Intramammary Infections..............................................................................................................................150

7.4 Shedding Patterns of Naturally Occurring Staphylococcus aureus Intramammary Infections Sampled Weekly Throughout Lactation.............................................151

7.5 The Effect of Strain Type on Cure-Rates in Dairy Cows with Naturally Occurring Staphylococcus aureus Intramammary Infections..............................153

BIBLIOGRAPHY .................................................................................................................................................................................155

APPENDIX A: ASSESSING AGREEMENT OF THE DE LAVAL DIRECT CELL COUNTER WITH SOMACOUNT300 ON QUARTER MILK SAMPLES FROM DAIRY COWS..........................164
LIST OF TABLES

Table 3.1 - Number of quarter samples that cultured positive (≥1cfu/mL) or negative from quarters with and without SA IMI and associated Sensitivity and Specificity of microbiologic culture, using daily samples, weekly samples and the combined results of both studies using a 0.01mL inoculum ........................................77

Table 3.2- Number of quarter samples that cultured positive (≥1cfu/mL) or negative from quarters with and without SA IMI and associated Sensitivity and Specificity of microbiologic culture, using daily samples, weekly samples and the combined results of both studies using a 0.1mL inoculum ........................................77

Table 4.1- Days in milk, milk production in pounds, cfu/0.01mL, ln(cfu) and linear score (LS) for all quarters, by parity and by PFGE pulsortype ........................................96

Table 4.2- Correlation between percentage daily change in ln(cfu) and linear score ..................................................................................................................104

Table 5.1 Median cfu/0.01mL, linear score (LS), and composite test day LS (Test Day LS) for every quarter and the number of samples culture positive for SA for each quarter using a 0.01 and 0.1mL inoculum ........................................128

Table 5.2 Mean, median and standard deviation over all quarters and by Parity and PFGE pulsortype for milk production, bacterial counts (cfu/0.01mL), log transformed cfu, linear score, composite test day results and linear score of composite test-day values ..................................................................................................129

Table 6.1 Days in Milk and Somatic Cell Counts of SA quarters and cows .................146
LIST OF FIGURES

Figure 4.1. Daily bacterial counts of SA (cfu/0.01mL) .........................................................97

Figure 4.2. Lowess smoother of ln(cfu/0.01mL) for each quarter over a 6.5 day window for Season 1-Winter ...............................................................98

Figure 4.3. Lowess smoothing curves of ln(cfu/0.01mL) over three separate 21 sample periods evaluated over a 6.5 day window ....................................................99

Figures 4.4. Lowess smoother for ln(cfu)/0.01mL over a 6.5 day window for the first 21 day sample period, Season 1 (Winter), in 2 quarters of cow 884-L.....................100

Figures 4.5. Lowess smoother for ln(cfu)/0.01mL over a 6.5 day window for the first 21 day sample period, Season 1 (Winter), in 2 quarters of cow 970-T.....................101

Figure 4.6: Daily cfu/0.01mL and Linear Score (LS) for each SA infected quarter over the first 21 sample period.................................................................102

Figure 4.7. Daily cfu/0.01 mL and linear score (LS) for quarters sampled over 3 seasons (Winter-Spring-Summer)...............................................................103

Figure. 4.8 PFGE of representative isolates from each SA infected quarter.............105

Figure 4.9. Daily ln(cfu)/0.01 ml by PFGE pulsotype for each 21 sample period........106

Figure 5.1 Lowess smoother graphs for individual SA quarters sampled from 26 to 44 weeks ..................................................................................130

Figure 5.2 Lowess Smoother at over 8 week periods for each quarter of cow 1384 (PFGE pulsotype 3).............................................................................131

Figure 5.3 Weekly shedding (cfu/0.01mL) of SA for Cow 101...........................131

Figure 5.4 Weekly shedding (cfu/0.01mL) of SA for Cow 115.............................132
Figure 5.5 PFGE of representative isolates from each SA quarter sampled ≥ 36 weeks in addition to including isolates from quarters that cured (-C) over the dry period...

Figure 5.6 Lowess Smoother panels over 8 week periods for each PFGE pulstype

Figure 5.7. Histogram of the frequency of test-day SCC expressed as a linear score by PFGE pulstype

Figure 6.2 Dendrogram of each isolate from treated cows
CHAPTER 1

INTRODUCTION

1.1 The Dairy Industry Today

Since the first regular shipment of milk by rail into New York City in 1841, the dairy industry has experienced remarkable growth and change. From refrigerated tankers and sexed semen to robotic milking machines, today’s dairy industry seems a distant cousin to the hand-milking farmer of old. With over 186 billion pounds of milk processed through over one thousand plants annually, milk has become a $35.4 billion dollar industry representing 30.2% of the total value of United States animal agriculture production.(USDA, 2008) Such dramatic change has brought many challenges, not the least of which has been ensuring the quality of the marketed product. Producing an average of 20,000 pounds of milk per lactation, today’s dairy cow is a metabolic powerhouse producing 3.7 times as much as her cousin circa 1975.(IDFA, 2008; USDA, 2002) Accomplished through genetic selection, improved nutrition and housing and the application of biotechnology, the industry has made remarkable strides in production per cow. Along with production, milk quality has experienced necessary improvements. Improved milk-handling equipment in addition to regulatory actions establishing standards of quality, sanitation, disease monitoring and eradication have helped and continue to provide the world with a nutritious and safe food supply.(USDA, 2002) Yet the quality and safety of milk, its products and the welfare of animals in production
systems are increasing concerns to the dairy-industry and public alike. Focus of these concerns are many and range from the impact of the quality of milk on processing, marketing and public health to conflicting views concerning the treatment of animals in production systems. For instance, milk produced from an inflamed mammary gland has lower levels of fat and casein with increased levels of whey protein and sodium among other constituents, such alterations in composition having negative affects on both the yield and quality of dairy products. (Blosser, 1979) In addition, debate persists within the dairy industry over current quality standards in the United States which limit the industries’ ability to capitalize on export markets to countries with higher quality standards. (NMC, 1998) Although the United States can boast one of the safest food supplies in the world, the growing global economy and recent terrorist events place the United States at increased risk for food-borne illness, of which milk is a potential source. (Oliver, Jayarao, & Almeida, 2005) Taking such factors under consideration, the quality and safety of milk quickly becomes a major concern.

A primary contributor to milk quality is udder health which, when compromised, is often manifested in high somatic cell counts (SCC). The most common cause of a high SCC is an intramammary infection (IMI) or mastitis, a subclinical or clinical inflammation or bacterial infection of the mammary gland. (Harmon, 1994) As many of these bacteria and their associated toxins are potential causes of human illness, *Escherichia coli* and *Staphylococcus aureus* (SA) for example, the need for control and prevention of such infections is paramount. (NMC, 2005) Given that the percentage of cows reported with clinical mastitis has increased from 1996 to 2007, it is clear that much
The goal of this literature review is 3 fold:

(1) To provide an appropriate backdrop to my research by examining our current understanding of mastitis, its prevalence, the impact of mastitis on the dairy industry and cows, and current strategies and associated pitfalls of detection modalities.

(2) Specifically review our current understanding of SA, its epidemiology, treatment and prevention.

(3) Finally, to outline the steps necessary for a more complete description of the epidemiology of SA, so we may progress toward a better understanding of S. aureus, its impacts and our ability to manage it.

1.2 Mastitis

Despite improvements in management, prevention, treatment and vaccines, mastitis continues to be one of the most prevalent and costly diseases of the dairy industry.(USDA, 2007) A complex disease with numerous causes, varied physiologic responses and outcomes, mastitis presents a unique challenge to veterinarians. Although there are viral and non-infectious causes of mastitis, the focus of this literature review will be mastitis due to bacterial infection as it is, by far, the most common etiology.

Bacteria that cause mastitis are grouped into two broad categories according to their impact on milk composition. Major pathogens which include SA, Streptococcus agalactiae, coliforms, streptococci and enterococci of environmental origin have the greatest economical impact with the greatest increase in SCC.(Harmon, 1994) Other pathogens are considered minor, resulting in modest increases in SCC and often
subclinical, having little effect on milk production. Of the major pathogens, SA is contagious with variable clinical outcomes resulting in infections that are often chronic, often subclinical and with variable effects on SCC.

1.2.1 The Physiology of Mammary Gland Inflammation

The fact that the bovine mammary gland is in constant contact with potential pathogens yet experiences relatively few incidences of mastitis, illustrates the remarkable efficacy of its inflammatory response. (Burton & Erskine, 2003) Compared to other tissues, the mammary gland must recruit a great many more immune cells in its defense to overcome the negative effects of dilution, milk fat and casein. (Burton & Erskine, 2003) Differences in a cow’s ability to effectively recruit neutrophils to the mammary gland may explain varied outcomes of bacterial challenge and are being explored. Mammary glands experiencing rapid neutrophil recruitment are more likely to experience spontaneous cures, while those with inadequate immune response are likely to develop acute or chronic mastitis. However, the mere presence of neutrophils in the mammary gland is insufficient for protection. The effectiveness of the bovine neutrophil is dependent on the presence of pre-existing immunoglobulin-G2 (IgG2) responsible for the coating and opsonization of bacteria providing more effective phagocytosis by neutrophils. Unfortunately, the production of IgG2 requires a specific immune response from either repeated exposure (as seen with repeated vaccination) or severe infection. Such exposure is often difficult to achieve. In addition, neutrophils must also undergo specific processes known as margination, migration, phagocytosis, respiratory burst and degranulation to be effective. The first two steps are critical in immune surveillance and
containment, the later responsible for the killing of pathogens. Margination and migration are complex processes, dependent on multiple cellular signals that, if disrupted, may result in severe mastitis and even death. Ironically, such disruptions are a normal part of bovine physiology. It has been established that the incidence of clinical mastitis is greatest at the time around dry-off and the periparturient period. (Vangroenweghe, Lamote, & Burvenich, 2005) The relationship between these two specific periods in lactation and the incidence of mastitis are due, in part, to the natural physiology of the mammary gland. While the type of immune response required for effective mammary defense is driven by a T-helper-1 mediated immune response, it has been well documented that the periparturient bovine immune system is dominated by a T-helper-2 response (TH2). (Burton & Erskine, 2003) Although a natural occurrence leading up to parturition, TH2 domination of the immune system may also be induced with external stressors such as heat, pain, stress or the administration of corticosteroids, each resulting in an increased risk of mastitis. (Burton & Erskine, 2003) It is in these instances when the immune response is muted, the recruitment of neutrophils and other immune effectors (opsonizing antibodies) are disrupted that the integrity of the mammary gland is compromised, increasing the risk of bacterial infection. In addition to periparturient domination with a TH2 driven immune system, estrogen and progesterone, the hormones necessary for proliferation of mammary epithelium, also undergo great flux during the periparturient period and are suspected to decrease the effectiveness of neutrophils. Fluctuations in glucocorticoids necessary for parturition, also have negative effects on the killing capacity and production efficiency of neutrophils. (Vangroenweghe, Lamote, & Burvenich, 2005) Recent studies have examined the effectiveness of neutrophils in the
udder environment. Mehrzad et al. examined neutrophil phagocytosis and killing of *SA* in blood and milk, reporting significant differences dependent on the milieu in addition to an increased performance from neutrophils isolated from primiparous cows versus multiparous cows. (Mehrzad, Duchateau, & Burvenich, 2009) The significance of this finding remains unclear and deserves much attention.

While research continues to explore the variable effectiveness of the bovine neutrophil, the basic physiologic and genetic factors and molecular mechanisms that influence the susceptibility of a mammary gland to mastitis are no better understood. (Burton & Erskine, 2003) Wilson found that multiparous cows with higher milk production were at increased risk of mastitis. (Wilson et al., 2004) Morse et al. also noted that the temperature-humidity index had a positive affect on the occurrence of mastitis, demonstrating that clinical mastitis increased more than 50% above the annual incidence following high temperature-humidity values. (Morse et al., 1988) Others have focused on genetic selection, selecting cows with comparatively low composite SCC (<50,000 cells/mL) as a potential indicator of mastitis resistance. The eventual affects of such an approach is worrisome as these cows may, in fact, be more susceptible to mastitis compared to cows with higher, but still healthy, SCC levels (100,000-200,000 cells per mL). Great potential may lie in the selection of cows with greater resistance to mastitis rather than the actual number of SCC, but the means to qualify or quantify such resistance remains unclear. Understanding the genetic basis of mastitis susceptibility within the context of the bacteria responsible for the disease will reveal new paths by which the immune function of the cow and mammary gland may be optimized to protect itself from bacterial infection.
1.2.2 The Prevalence of Mastitis

The percentage of cows in the United States with clinical mastitis was 16.5% in 2007 (2% greater than lameness), while 23% of cows were reportedly culled due to mastitis. (USDA, 2008) The reported herd level prevalence of SA in North America and Europe has ranged from 31% to 100%. (Lam, 2008) To affect change in any of these statistics it is essential that we understand the specific impacts of each mastitis pathogen on the cow and management of the herd.

The hallmark of the epidemiological triangle, the incidence of mastitis lies at the intersection of the resistance of the cow to IMI and host ability to self-cure, the environment (exposure to pathogens/management), and the pathogen itself. Therefore, the incidence of mastitis varies greatly between farms and, at times, within farms depending on season, bedding, housing availability, milking machine maintenance and a host of other management factors. Olde Riekerink et al. demonstrated a seasonal influence on mastitis examining 300 Dutch dairy farms. (Olde Riekerink, Barkema, & Stryhn, 2007) Describing peaks in July in both a contagious pathogen (SA) and an environmental pathogen (E. coli), the report highlights the possible seasonal effects on pathogens such as SA long considered to be moved solely from cow to cow. (Olde Riekerink, Barkema, & Stryhn, 2007) Østerås et al. also noted a similar seasonal effect in quarter samples collected from Norway. (Osteras, Solverod, & Reksen, 2006)

As we try to build a better understanding of mastitis and SA IMI in particular, it is important to recognize that there are many limitations to our progress, much of which is the focus of this review. For instance, much of the field research conducted has relied on mastitis screening conducted by farmers thus limiting infections studied to those that
present with clinical signs. (de Haas, Veerkamp, Barkema, Grohn, & Schukken, 2004; Green, Schukken, & Green, 2006; Olde Riekerink, Barkema, & Stryhn, 2007) Such limitations make it difficult to compare mastitis incidence or the effectiveness of treatment and control strategies across herds. It is the intent of this review to make it clear that fundamental questions about mastitis diagnostics be addressed before we can truly understand the epidemiology of pathogens such as SA, and that such understanding is paramount in developing effective and efficient control programs.

1.2.3 The Economic Impact of Mastitis

On a herd level, the impacts of increased Bulk Tank SCC (BTSCC) are significant. Ott et al. reported in 2001 that herds with a low BTSCC ( < 200,000 cell/mL) produced 4.6% more milk per cow per year than herds with medium BTSCC (200,000 to 399,999 cells/mL) and nearly 12% more than herds with a high BTSCC (≥400,000 cells/mL). This translated into a profit of $103/cow per year more for herd with a low BTSCC compared to herds with a BTSCC between 200,00 and 399,000 cells/mL. (Ott & Novak, 2001) When compared to other diseases encountered on dairies, mastitis represents the most costly, weighing in at 26% of the total. (Lightner, Miller, Hueston, & Dorn, 1988) Dependent on cow traits, stage of lactation, current milk price, cost of replacements, labor and veterinary care, estimates of the cost of clinical mastitis has been calculated at 6% of the value of production; at 2007 prices that is upwards of 2.1 billion dollars. (Bar et al., 2008; Janzen, 1970; Shim, Shanks, & Morin, 2004) Adding to this staggering figure is an estimate of the cost of sub-clinical mastitis (loss of premiums, milk production and increased replacement cost) which was reported at $960
million. (Wells, Ott, & Seitzinger, 1998) In total, it is estimated that 70-80% of a 
projected $140 to $300 per cow/year loss is in fact due to subclinical mastitis. (Gill, 
Howard, Leslie, & Lissemore, 1990) It has been reported that quarters with clinical or 
subclinical mastitis experience a variable drop in milk yield from 5 to 24%. (Janzen, 
1970) Although the exact difference between the SCC of healthy and uninfected quarters 
remains up to debate, the impact of elevated SCC is well established. Hortet and Seegers 
reported a 0.5 kg decrease in milk yield for every two-fold increase in SCC (Hortet & 
Seegers, 1998) while others demonstrated a 5% drop in milk production for every 
additional 100,000 cells/mL. (Hillerton & Berry, 2005; Seegers, Fourichon, & Beaudeau, 
2003) Such losses have greater impact considering that such losses are not recovered 
completely in the current or proceeding lactation. Wilson calculated a loss of 1200 kg for 
the remaining lactation in multiparous cows and a 700 kg loss for primiparous cows. 
(Janzen, 1970; Wilson et al., 2004) Studies specifically evaluating milk yield in SA 
infected glands revealed a difference in production over 15 kg. (Janzen, 1970) However, 
others have demonstrated that whilst production decreases by 20% in the infected quarter, 
there is compensatory production of 13% in normal quarters decreasing overall 
production by less than 10%. (McDougall, 2002)

The effects of mastitis reach beyond the bulk-tank. Ott et al. revealed that herds 
with low BTSCC (< 200,000 cells/mL) not only produced more milk, they produced 
6.2% more calves in a year. (Ott & Novak, 2001) As far back as 1979, Blosser supposed 
that with a 2% per year reduction in loss from mastitis for 10 years, the benefit to cost 
ratio (of associated mastitis research) would be 9.6 to one. (Blosser, 1979) Why then has 
the rate of clinical mastitis increased from a reported 13.4% to 16.5% over the last 10
years? (USDA, 2008) While some suppose that our rate of detection has increased rather that the incidence, others contend that it is the nature of mastitis that has changed, presenting with more detectable, clinical cases.

Interestingly, although the cost of mastitis represents a direct monetary loss to producers on a cow by cow basis, Losinger reported that when considering larger scale economics, that it is consumers that stand to gain from BTSCC’s less than 200,000 cells/mL. If such a decrease in SCC were to happen, Losinger predicted that the US Dairy industry actually stands to lose $20.6 billion in milk production value. These estimates were based on economic principles of the elasticity of supply and demand and the assumption that a BTSCC of <200,000 cells/mL would result in an increase in production of more than 72 billion kg thereby decreasing the market price via transfer of economic surplus to consumers. (Losinger, 2005) Whether such predictions would translate in reality remain to be seen.

1.2.4 Mastitis and Public Health

The potential transmission of pathogens, including Salmonella sp. and Listeria, is a legitimate concern. (Oliver, Jayarao, & Almeida, 2005) A survey of United States bulk tanks in 2000 was conducted and found that 26.7% of bulk tanks contained one or more species of pathogenic bacteria including E.coli, Salmonella and Listeria. (Jayarao & Henning, 2001) Although minimized by pasteurization, the risk of human disease persists when pasteurization fails or is intentionally avoided. Indeed, consumption of unpasteurized milk, particularly that originating from high SCC cows, poses substantial risk to public health. Associated with several outbreaks, the consumption of raw or
inadequately pasteurized milk has been implicated in 46 outbreaks of human disease in the United States between 1973 and 1992. (Jayarao & Henning, 2001) The emergence of multiple antibiotic resistant strains of *Salmonella ser. Typhimurium DT104* add to the concern for both farm employees and the public as treatment options by those affected are then limited.

Although there may be little concern related directly to the consumption of pasteurized high SCC milk, indirect public health issues related to bovine mastitis warrant consideration. Mastitis constitutes the majority of antibiotic use on dairy farms, accounting for half of all antibiotic used on Canadian dairies. (McCarron, Keefe, McKenna, Dohoo, & Poole, 2009) Reports are consistent in illustrating that there is a positive relationship between bulk tank SCC and violative antibiotic residues. (Ruegg & Tabone, 2000) Approximately 80% of violative residues in milk can be traced to either lactational or dry cow mastitis treatments. (Schukken, Wilson, Welcome, Garrison-Tikofsky, & Gonzalez, 2003) Reports from Michigan’s Department of Agriculture revealed that 90% of violative residue detected in bulk tank milk were the result of antibiotics used to treat mastitis. (Erskine, Wagner, & DeGraves, 2003) As consumption of dairy products containing antibiotics by persons allergic to certain antibiotics may prove fatal, such correlations highlight the potential impact of udder health beyond the farm.

1.2.5 The Impact of Mastitis on Cow Welfare

Definitions of welfare are varied and inconsistent. The most useful definition was put forth by Broom and describes animal welfare as “the state of an animal in its attempt
to cope with its environment.” (Broom, 2004) Currently, the agricultural community has placed animal welfare within it’s top three concerns next to environmental issues and food-safety. (Rollin, 1995) When viewed from this angle, it becomes more evident that the effects of mastitis reach far beyond the bulk tank. Intramammary infections with organisms such as *E. coli* and *SA* resulting in either septicemia or the necrosis of mammary tissue can indeed be life threatening. Although it is easy to appreciate the negative effects of mastitis on a cow’s welfare when infections are severe and life threatening, such cases are comparatively infrequent on well managed dairies. Equally important are the negative effects of subclinical and chronic IMI’s on cow welfare. The vast majority of IMI’s are either subclinical or mild, a minority resulting in the cessation of lactation of the individual quarter. Not discounting the immediate impact mastitis has on the welfare of individual cows, for the pain associated with inflammation in even mild clinical cases are certainly significant to the animal, the long tem effects of mastitis on cow welfare are well documented in comparison. Ahmadzadeh et al. demonstrated that cows with a recorded mastitis event experienced lower reproductive efficiency, an experience necessary to remain in the dairy system. (Ahmadzadeh et al., 2009) Seegers at al. also established that cows experiencing either a mastitic event or an increase in SCC were at an increased risk by a factor of 1.5 to 5 for culling, events occurring early in lactation posing the greatest risk. (Seegers, Fourichon, & Beaudeau, 2003) If one accepts that premature culling/death is indeed contrary to one’s welfare, it is clear then that udder health has a major impact on the welfare of cows worldwide.
1.3 References


NMC. (1998). *Current Milk Quality in the U.S.*


CHAPTER 2

LITERATURE REVIEW – IMPEDIMENTS TO UNDERSTANDING THE EPIDEMIOLOGY OF

BOVINE STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS

2.1 Our Current Understanding of the Epidemiology of S. aureus Mastitis

2.1.1 The prevalence of S. aureus Mastitis

When discussing bacteriology in the realm of mastitis, Staphylococcal infections are divided into two very broad categories according to their source (contagious and environmental) and laboratory identification (coagulase positive staphylococci and coagulase negative staphylococci, or CNS). Staphylococcus aureus (SA), is the single gram positive, coagulase positive mastitis pathogen grouped into the contagious category. While CNS are considered opportunists, SA is thought to be spread primarily from cow to cow during milking (Sears and McCarthy, 2003). The most prevalent contagious mastitis pathogen in the United States, NAHMS reported that 52.3% of operations performing milk cultures at some point during 2007 detected SA. (USDA, 2008) Using single bulk tank samples to estimate prevalence on US Dairies, SA had the highest prevalence (43%) of all contagious pathogens irrespective of herd size. (USDA, 2008) In a study of 8 herds, over 4 geographic locations, the prevalence of SA ranged from 10% to 32%. (Middleton, et al., 2002a) Yet, in a second study by the same authors across 15 commercial dairy herds, prevalence of SA IMI (based on composite samples at freshening) ranged from 0 to
12.3%. (Middleton, et al., 2002b) The variable prevalence and persistence of SA within dairy herds is multifactorial, due in large part to biosecurity protocols (or lack thereof) and management practices on individual dairies as well as the very nature of SA itself. The persistence of SA IMI’s in the mammary gland is thought to be the result of the production of hemolysins and proteins which damage tissue and allow for intracellular colonization, making it possible to evade both the cows immune system and treatment, resulting in chronic infections. (Sears and McCarthy, 2003) In addition, SA may produce an extracellular polysaccharide capsule masking antibodies, allowing the bacteria to evade compliment thus avoiding phagocytosis. (Middleton, et al., 2006)

2.1.2 Sources and Transmission of S. aureus

As a contagious pathogen, the spread of SA has typically been associated with milking practices or faulty equipment in the milking parlor. Osteras et al. supported this theory, reporting that SA was more likely to be cultured from a quarter of a cow with another quarter also infected, indicating the transmission of SA is highest between quarters and within cows. (Osteras, et al., 2006) However, this limited view neglects the fact that it has been well established that heifers frequently freshen with SA IMI. Nickerson et al. examined the prevalence of mastitis in heifers, reporting that 13% to 37% of pre-partum samples contained SA. (Nickerson, et al., 1995) Fox et al. also reported in a multistate study of 28 dairies that 9% of pre-partum samples were culture positive for SA. (Fox, et al., 1995) Although only 3% of the quarters remained positive at calving, clearly, the spread of SA is not limited to the milking parlor, leaving other possible sources to be considered. Veterinarians regularly managing SA IMI in
veterinary practice may find that the epidemiology of SA appears to vary from herd to herd, spreading rapidly in some and slowly in others. Historically it has been presumed that the differences in the rate of infection were management dependent. However, Sommerhauser et al. (2003), studying differences in the effectiveness of control measures, reported that control may me more dependent on the strain of bacteria than the management practices of the farm itself (Sommerhauser, et al., 2003). Examining SA infections on seven dairies during a control program which included the use of individual towels, application of post-milking teat dip, proper milking technique and targeted treatment and culling, Sommerhauser et al. reported that four of the seven herds studied had SA IMI rates of 24-27%, each with a predominant PFGE pulsotype, while the three remaining had IMI rates of 4-12%, revealing several distinguishable PFGE pulsotypes. (Sommerhauser, et al., 2003) Sommerhauser et al. (2003) noted that while herds with several PFGE pulsotypes experienced a relatively higher rate of new infections, they appeared to have little tendency to spread from quarter to quarter and therefore benefited little from the control program geared toward the control of contagious pathogens. (Sommerhauser, et al., 2003) Similarly, in herds experiencing high transmission rates, Barlow et al. concluded that efforts focused on mastitis control practices and selective culling or drying of quarters would be most beneficial. (Barlow, et al., 2009) Understanding such important differences between strains will prove essential in developing SA control programs specific to individual operations. One important consideration should be made however, the control program instituted in the study recommended culling of cows with persistent SCC > 100,000 cells/mL with a SA IMI. Aggressive culling strategies such as this may certainly explain the overall decrease in
the percentage of infected cows reported across all of the herds in the study in addition to potentially targeting particular strains of SA prone to high SCC.

In addition to the effects of individual strains and management, the individual cow may also play an important role in the epidemiology of SA. Zadoks et al. examined risk factors for SA IMI, reporting that 100 new infections were detected in 22,593 quarter samples. (Zadoks, et al., 2001) In the study it was reported that that rate of SA infection was higher in bovine herpes virus-4 seropositive cows, in right quarters, in quarters exposed to the SA quarters of the same cow, in quarters that had recovered from SA and in quarters with severe teat calluses. (Zadoks, et al., 2001) It should be noted that only new infections were included for the purpose of this study and existing infections were excluded, which may limit the strain representation. Also, recovery from a SA infection required only one negative sample, which may falsely define strains that shed at low numbers as cured and re-infected rather than simply a persistent infection.

As our ability to control cow factors may be limited, efforts aimed at developing prevention strategies have also explored the potential sources of SA exposure. Several studies examining strain differences have been conducted attempting to identify potential sources of SA and explaining differences in infection patterns amongst herds. Potential sources of SA identified have been associated with milk, body sites and the environment, although to a much lesser extent. (Roberson, et al., 1998) Exploring possible sources, Sears et al. revealed that heifers with SA colonizing the skin of the teat were at 3.34 times greater risk of a being infected with SA at freshening. (Sears and McCarthy, 2003) In addition, Owens proposed that flies may be capable of transmitting SA. (Owens, et al., 1998) The potential of such transmission has since been substantiated by Gillespie et al.
who compared DNA fingerprints from heifer and fly isolates of SA.(Gillespie, et al., 1999) Capurro et al. explored the possible reservoirs for SA, comparing PFGE patterns of SA isolates obtained from environmental and body site swabs to those obtained from infected quarters.(Capurro, 2009) While some pulsotypes were associated strictly with milk, others were associated with milk and hock skin and stanchion surfaces. Zadoks et al. applied PFGE to SA isolates and found that the predominate genotype in the milking herd was different from heifer mastitis isolates, raising more questions about the potential sources of heifer infections.(Zadoks, et al., 2000) Zadoks et al. later examined more isolates from lactating cows (not heifers) and concluded that teat skin was not a significant reservoir for bovine SA IMI based on PFGE comparisons.(Zadoks, et al., 2002) Using MLST, Smith et al. further substantiated this finding examining a global population of SA, also reporting that milk isolates appear to belong to different strains than isolates from teat skin.(Smith, et al., 2005a)

2.1.3 Prevention of S. aureus

Recognized as a significant cause of chronic mastitis and monetary loss, several management approaches have been put forth to control or eliminate SA from dairy herds. An effective program requires timely identification of all cows infected with SA. Such screening includes regular monitoring of the bulk tank in addition to screening all cows as they enter the lactating herd. Although such practices are seemingly straight forward, they may be cost prohibitive or difficult to implement depending on financial, facility or management resources. Formal programs have included the 5 point program established in the 1960’s which eventually became the more extensive 10-Point program developed
by the NMC in 2001. (Barkema, et al., 2006) Private veterinary practitioners have also developed less formal programs, tailored to individual farms. What is common to all programs is an emphasis on proper milking procedures, post milking disinfectants, proper screening and biosecurity in addition to segregation and culling when possible. As discussed in Sec. 2.1.2, several studies have revealed that some strains of SA may be more effectively controlled or eliminated through segregation than others, highlighting the need for continued examination of the effects of strain differences on SA epidemiology. (Middleton, et al., 2001) At this time, for all that has been done to study SA IMI, we understand relatively little about its true epidemiology. Much of this lack of understanding is due in part to inconsistencies in research methods in addition to research based on experimental infections no longer believed to represent the natural infection we are trying to manage and prevent. For this and many other reasons, SA remains a significant problem in the dairy industry and will persist as such until a unified approach to research is assumed in addition to developing systems to allow for valid comparisons across herds as well as geographic regions. The next step then is to understand how current research methods have limited our understanding of SA IMI.

2.2 Diagnosing S. aureus Intramammary Infections

An effective control or eradication program requires timely and accurate identification of all cows infected with SA IMI. Of the three coagulase-positive Staphylococci, SA is the only species expressing hemolysis that is commonly found in bovine milk. (Boerlin, et al., 2003) However, up to 25% of SA isolates from bovine mastitis do not express the hallmark β-hemolysis. For this reason, hemolytic growth on
blood agar confirmed with a tube-coagulase test has been the accepted method of SA confirmation in mastitis microbiology. (NMC, 2004) However, confirmation of microbiologic identification is the single, relatively, consistent element in mastitis research. The proceeding section addresses specifically the manylimitations of our current knowledge regarding SA IMI particularly with regard to an absence of any consistent definitions for infection, method of culture, chronicity or cure. Such inconsistencies are pervasive throughout the literature, allowing for little comparability between studies, and therefore limit broad applications of findings. Such limitations have left veterinary practitioners to adapt published work to personal experience in developing management strategies. Although such adaptation has indeed proven effective for many veterinary practitioners, it is critical to endeavor to develop consistent methodology and specific inclusion criteria if we are to develop a true understanding of the epidemiology of SA IMI in an ever growing and global population.

### 2.2.1 Defining Intramammary Infection

As far back as 1973, a call was made for science to reach an agreement as to what actually defined “mastitis” in terms of cell count (SCC) or culture. (Neave, 1973) Neave pointed out then that the definitions of mastitis were “not suitable” and “cumbersome.” In addition, Neave noted that the current definition of a “normal” gland was too rigid. Neave (1973) proposed that bacteriological tests alone would suffice in diagnosing IMI’s when certain criteria where met, provided that the diagnosis was made on two successive samples and samples were collected by a trained technician according to established guidelines. (Neave, 1973) Neave’s criteria are outwardly appealing but when evaluated in
the context of our current understanding of mastitis they become less so. The supposition that bacteria are isolated by simple methods can be debated as the incidence of *Mycoplasma* mastitis continues to increase, isolation of which requires special media and incubation conditions. The detection of bacteria is indeed dependent on both the secretion of sufficient numbers of bacteria from affected quarters and the amount of inoculum used in testing. It has been well established that upwards of 30% of clinical quarters provide no recovery of bacteria using microbiological culture. Whether this same trend is expected in subclinical samples is difficult to evaluate, as we have no definition of subclinical IMI. Lastly, presuming that a 10-fold increase in SCC is present in an infected quarter places limitations on our evaluation of those infections established microbiologically with no evidence of elevated SCC. In contrast, Olde Riekerink recently defined a new *clinical* infection simply as one separated by at least 14 days of apparently normal milk. (Olde Riekerink, et al., 2007) However, as this definition is limited by the gross appearance of either the milk or the quarter, it neglects to consider the possible continued presence of bacteria once clinical symptoms have resolved. Depending on the outcome of interest, these definitions may or may not prove useful and at worst may prove to be very misleading.

2.2.2 Microbiological Diagnosis of *S. aureus* Intramammary Infections

It has been estimated that a mere 30% of subclinical *SA* infections become clinical. (Sol, et al., 2000) Taking such estimations under consideration, it is evident that the control and management of *SA* IMI is dependent on an accurate understanding of and therefore dependant on accurate diagnosis of subclinical *SA* IMI. For this reason,
diagnostic and screening methods used must be consistent to allow for comparison across studies.

Indeed, the success of any mastitis control program lies in the ability to detect subclinical infections. Yet such success is dependent on a definition of infection that is inconsistent and impractical. The FDA’s published guidelines for the collection of efficacy data of mastitis treatments require recovery of the same organism from at least 2 milk samples collected separately.(Postle, 1976) Other microbiological definitions for an IMI commonly used include: The presence of bacteria in two of three consecutive (different days) cultures; or the presence of the same species of bacteria on duplicate samples (2 samples collected on the same day, one after the other). However, there have been numerous other definitions employed with regular deviations within studies (Borm, et al., 2006) where a positive culture from a single sample was deemed sufficient.(Borm, et al., 2006, Dingwell, et al., 2002, Dingwell, et al., 2003, Erskine, et al., 2003, Fox, et al., 1987) As evidenced in a meta-analysis published in 2002, there has been little consistency in defining either the number of samples or colony forming units (CFU) required to define an IMI.(Djabri, et al., 2002) The meta-analysis revealed 14 different definitions of infection based on CFU and sample frequency. It is currently supposed that reliable results require repeated sampling of individual quarters which may be cost prohibitive.(Graber, et al., 2007) Definitions specifically addressing SA IMI have also varied tremendously throughout the published literature. Daley defined a SA IMI as one characterized by viable bacteria in the milk and an elevated SCC.(Daley, et al., 1991) In contrast, cows included in a treatment trial by Oliver required two positive quarter samples 1 week apart in addition to a SCC >400,000 cells/ml, while Deluyker et al.
required a single positive culture and a SCC greater than or equal to 300,000 cells/mL. (Deluyker, et al., 2005, Oliver, et al., 2004a) Buelow et al. defined the gold standard as the isolation of two or more colonies of SA on two or more occasions out of six consecutive daily samples. (Buelow and 1996)

Perhaps the one agreed upon procedure is that of the method of sample collection. As sterile collection of milk samples is at best impractical, aseptic sampling practices are necessary. To that end, the NMC has published guidelines for collecting milk samples for both microbiological and somatic cell count evaluation. (NMC, 1999) However, what is sampled (composite or quarter), when it is sampled (pre or post milking) and how much is used for culture (0.1 ml or 0.01ml) and what type of microbiological results imply infection remains up for debate. Buelow et al. noted in 1996 that the standardization of milk sample collection and culture methods would make the investigation of factors such as bacterial strain much easier. (Buelow, 1996) As of yet such an agreement has never been accomplished, providing little if any continuity or comparability between published works. Limitations in developing such standards include noted variability in the shedding of organisms including SA. Developing a clear understanding of these differences is critical in building continuity amongst researchers.

2.2.2.1 The Effects of Variable Bacterial Shedding on Diagnosis

The extent to which the shedding of SA effects its transmission has been difficult to evaluate as study methods have varied throughout the literature. In one of the most comprehensive papers published on the diagnosis of mastitis by bacteriological methods, Neave examined 206 SA infected quarters up to 20 times. (Neave, 1973) Neave (1973)
reported that 44% of quarters with established SA infection were culture negative at least once during the sampling period. Unfortunately, the report offered little in regards to explaining the frequency of sampling and the time period over which samples were collected, therefore the application of the data is limited. (Neave, 1973) In what has been probably the most frequently cited and misquoted published report, Sears et al. described the shedding patterns of SA in bovine IMI’s. (Sears, et al., 1990) Sears et al. examined 21 quarters of seven cows experimentally infected with the Newbould strain of SA over 28 days in addition to four naturally infected quarters in four cows over 16 days. (Sears, et al., 1990) Samples collected from experimental infections in the study were taken after milking, while samples from naturally infected cows were taken after milking in the a.m. and prior to milking in the p.m. Samples collected from natural infections were cultured using 0.05ml, while samples from experimental infections were plated using 0.1 ml. Colony counts were recorded up to 3500 cfu (350 cfu/0.1mL), and considered positive with at least 1cfu/0.1mL inoculum yet required greater than 1 cfu to be considered positive using a 0.05 mL inoculum. Sears et al. (1990) concluded that SA was shed in a cyclical manner and therefore consecutive samples were necessary for accurate diagnosis. Shedding cycles from the study were described as “low” (mean of ≤ 1000 cfu/mL) and “high” (mean of ≥ 2000 cfu/mL). Whether or not the use of median values would have changed this interpretation has not been explored. Of the 4 natural infections examined by Sears (1990), 2 exhibited a “low” shedding cycle, 1 culturing positive 93% of the time (cultured negative 2 out of 29 samples), and the second culturing positive 63% of the time (18 of 29 days). From Sears’ work on experimental and natural infections, Sears suggested that 40% of SA IMI would be categorized as “low shedders.” Daley et al.
(1991) also reported a cyclic rise and fall in CFU in experimental infections using the Newbould strain and a concomitant but asynchronous cycling of SCC. (Daley, et al., 1991) In contrast, Buelow reported incidences of “low shedders” from 45% in one unpublished study and 65% from another set of unpublished data. (Buelow, 1996) An important consideration is the effect of pre versus post milking sampling. Sears et al. reported in 1991 that, although neither sampling time was neither perfectly sensitive nor “roughly comparable”, the sensitivity of pre-milking samples for SA detection was 91% while post-milking samples had a sensitivity of 81%. (Sears, et al., 1991) It was also not clear in Sears’ study (1990) whether or not the samples had been frozen. Godden et al. compared sample methods in addition to the effects of freezing. (Godden, et al., 2002) The report concluded that fresh samples taken post-milking were inferior to pre-milking samples that were either fresh or frozen or post-milking samples that had been frozen. (Godden, et al., 2002) Since the original report by Sears (1990), there have been variable interpretations of the reported “cyclicity” resulting in references to “sporadic” or “intermittent shedding” implying that SA is frequently not shed at any level by SA infected glands. (Djabri, et al., 2002, Graber, et al., 2007, ten Napel, et al., 2009) Such interpretations have illogically resulted in developing sample strategies requiring duplicate and multiple samples. However, if one were to accept that SA was shed intermittently it would seem more sensible to require a single positive sample to rule in infection, and multiple negative samples to rule out a SA IMI. Whether or not multiple samples are truly necessary remains to be proven.

2.2.2.2 Sample Strategy
NMC’s published guidelines suggest that 2 of 3 consecutive cultures should be positive to be considered infected. (NMC, 2004) Comparing duplicate versus consecutive sampling, Postle reported that the disagreement between consecutive and duplicate samples (2–7 days apart) of 1.6% and 2.7%, respectively. (Postle, 1976) However, it should be considered that Postle introduced the nature of the “instability” of infections due to high self-cure rates and the fact that such spontaneous recoveries could not be differentiated from streak canal contaminants in consecutive samples. Buelow et al. defined the gold standard as the isolation of two or more colonies of SA on two or more occasions out of six consecutive daily samples. (Buelow and 1996) Erskine, however, reported that percentage agreement between duplicate samples was highest for the contagious pathogens such as SA (94.2%), suggesting that a single quarter sample might be adequate in determining infection status. (Erskine and Eberhart, 1988) An important consideration to be made when evaluating such work is appreciating that agreement does certainly not imply infection, as a single colony was sufficient to define any type of infection, major or minor. Recently, Anderson et al. proposed a “gold standard” in the microbiological evaluation of a single sample which was based on 3 consecutive weekly milk samples requiring two positive cultures (not on test day), regardless of SCC. (Anderson, 2009) The impact of colony count and SCC on the definition of infection will be discussed in later sections of this review.

Although techniques requiring duplicate or repeated samplings are designed to maximize test sensitivity and specificity, on a large scale, they are often impractical or too costly. In an attempt to circumvent the cost of quarter sampling, some have used composite samples, which, although economical, have often met with objection by those
insisting on repeated quarter level evaluation. (Fox, et al., 1987) Sobreira used composite milk samples, requiring ≥5 cfu on duplicate samples to diagnose an IMI. (Sobreira, 2009) Buelow et al. also considering quarter level samples impractical and costly, evaluated composite sampling and reported that 2 repeated composite samples (at three day intervals) were 100% sensitive for SA IMI. (Buelow and 1996) However, it is important to consider that the population used in Buelow’s study was a single herd with a history of SA and therefore strain diversity, in addition the proportion of chronic versus new infections, may have had an effect on sensitivity and specificity of diagnostic schemes.

2.2.2.3 The Effects of Inoculum Volume on Identifying S. aureus Intramammary Infections

Adding to the confusion in comparing published reports, there is further inconsistency in the amount of milk subject to microbiological evaluation. Volumes of 10, 50 and 100 microliters have been employed, all affecting the sensitivity with which pathogens will be isolated. (Dingwell, et al., 2002) While a standard inoculum of .01mL of milk is often used, others have increased inoculum volume to 0.05mL and 0.1mL to increase the sensitivity of the microbiologic screening. (Middleton, et al., 2001, Oliver, et al., 2004a, Oliver, et al., 2004b, Sol, et al., 2000) Such variation exists in spite of recommendations by the NMC to use a 0.01 mL inoculum for the isolation of bacteria from milk, while some have used multiple different inoculums (0.01 mL, 0.03 mL and 1.0 mL) within the same study. (Middleton, et al., 2006) Buelow et al. published a report in 1996 evaluating the optimal inoculum volume for the detection of SA IMI. (Buelow, 1996) Buelow (1996) concluded that single and repeated quarter milk samples cultured
using a 0.1mL inoculum had sensitivities of 86 to 87% and 94 to 99%, respectively. An important consideration in evaluating the application of Buelow’s findings (1996) is that the study was based on 54 quarters experimentally infected with the Newbould strain of SA. In addition, based on the report by Sears (1990), an assumption that SA was shed intermittently was used in developing models to evaluate the effect of inoculum volume. Also, the probability function used for the length of a shedding cycle in Buelow’s study was based on 15 experimental SA IMI using the Newbould strain. Buelow et al. later combined the results of several studies attempting to identify the optimal milk inoculum volume, reporting that using a volume of 0.1 mL rather than 0.01 mL had the greatest effect on sensitivity on composite sampling strategies and that using 0.01 mL resulted in a larger range of sensitivities for all collection strategies. (Buelow, 1996) It should also be noted that this report combined data from several authors, including those obtained from experimental infections using the Newbould strain of SA, therefore creating questions about the comparability of the results to those with natural infections.

2.2.2.4 The Effects of Colony Count in Defining Infection

Just as there is no standard inoculum used in mastitis diagnostics, neither is there a standard colony count used to define infection. Colony counts used to define infection have ranged from 1 to 3 to 10 and even 50 cfu/0.01 mL, again, making comparisons of results across studies difficult at best. (Dingwell, et al., 2002, Dingwell, et al., 2003, Fox, et al., 1987, Pantoja, et al., 2009) With regard to SA specifically, Oliver required a single colony of SA to be present in duplicate samples prior to treatment and a single colony from any one of three separate samples post treatment to be positive, while Owens wrote
that the presence of any SA colonies was a positive in a heifer treatment trial. (Oliver, et al., 2004b, Owens, et al., 2001) Others have required a single cfu per 0.01 mL of milk on a single quarter sample to diagnose a SA infection (Dingwell, et al., 2006), some have required ≥500 cfu/mL or ≥100 cfu/mL within the same study (Haveri, et al., 2008) and others have relied on repeated composite (Middleton, et al., 2002b) or quarter samples, still others have offered no definition for infection at all in the published report. (Dingwell, et al., 2003, Dingwell, et al., 2006, Middleton and Fox, 2001, Middleton, et al., 2002b, Oliver, et al., 2004a, Owens, et al., 1988)

Recently Torres et al. examined the utility of colony count in assessing IMI status, finding that IMI could accurately be diagnosed with a single quarter level sample, and that the required number of cfu for a positive diagnosis was dependent on the type of pathogen identified. (Torres, et al., 2009) Torres et al. (2009) determined that a cut-off of 100 cfu/mL was appropriate to diagnose a SA IMI on a single sample in comparison to other pathogens requiring a 1,000 cfu/mL cut off. (Torres, et al., 2009)

2.2.3 Defining Chronicity

The definition of chronicity has also varied dramatically between published works, if any definition was offered at all. Defined by Dinsmore et al. as a quarter which had SA isolated from milk samples at least twice during a herd monitoring scheme, by Owens as an IMI that had persisted greater than 4 weeks in experimental infections, by Petitclerc as an IMI present for 15 days and again by Oliver as an IMI with at least 100 cfu/mL of the same organism isolated pre-partum and at any of 3 points after calving reveals a serious flaw in current mastitis research: presuming the duration of
infection. (Dinsmore, et al., 1995, Oliver, et al., 2004b, Owens, et al., 1997, Petitclerc, et al., 2007) It has been a presumption in many treatment trials, particularly in pre-partum heifers, that the SA isolated from the pre-partum samples is identical to the post-calving isolates. To date, there has been no study that has documented the status of all quarters of SA infected cows from the beginning to the end of lactation, or from one lactation to the next, monitoring the infections status. It has been presumed that cows screened mid-lactation with two or more infected quarters, began with a single infected quarter with other quarters infected later.

2.2.4 Diagnosis of *S.aureus* Using Somatic Cell Counts

Milk SCC’s have long been viewed as an indicator of udder health and milk quality and are the major indicator in monitoring programs aimed at improving udder health. (Fetrow, et al., 1988) The SCC represents leukocytes secreted into the milk as an essential part of the udder’s immune defense in addition to a small percentage of epithelial cells. An uninfected mammary gland secretes milk with relatively few cells, consisting mostly of macrophages and lymphocytes. However, in the face of infection there is a precipitous increase in the number of neutrophils within a matter of hours in excess of $10^6$ cells/mL. (ten Napel, et al., 2009) While some report that a healthy udder should have a SCC less than 100,000 cells/mL, the NMC reports that milk from uninfected quarters will have a SCC of less than 200,000 cells/mL, the probability of infection increasing along with SCC. (Hillerton and Berry, 2005, NMC, 1999) Pantoja et al. recently compared infection status predictions using a SCC cut-off of 200,00 cells/ml. (Pantoja, et al., 2009) Pantoja et al. reported a sensitivity of 0.64 for the
detection of IMI using quarter samples, and a sensitivity of 0.47 using composite test day information. Pantoja et al. also reported that to achieve similar sensitivity for detecting subclinical IMI’s at the composite level, a breakpoint of 100,000 cells/mL would need to be used. This is in agreement with Pyorala who reported that the SCC for the composite milk from cows should not exceed 100,000 cells/mL. (Pyorala, 2003) In contrast, Schukken reported that a cut-off of between 200,000 and 250,000 cells/mL was an optimal operational threshold to reduce diagnostic error (Se 75% and Sp 90%), highlighting that such a cut-off was not necessarily reflective of optimal udder health, rather optimal for diagnostic efficiency. (Schukken, et al., 2003) In an attempt to simplify interpretation and comparisons with losses in milk production, SCC has been commonly expressed as a “linear score” (LS), the SCC modified by base 2. (Fetrow, et al., 1988) As LS is a direct reflection of SCC, LS is highly correlated with production losses and the presence of IMI in addition to cheese yield and remains the most common method of reporting SCC’s. Although a LS of 5 (283,000 cells/mL) is suggested to be a proper threshold when examining records for mastitis control, as SCC reports are typically at the cow level, little is known about the utility of such thresholds addressing quarter level impact of specific mastitis pathogens. (Reneau, 1986)

When using SCC to evaluate udder health, other factors should be considered. The sensitivity, specificity and prevalence of IMI must be considered when evaluating the predicted value of SCC, meaning that such judgments must be made in the context of the individual herd. (McDermott, et al., 1982) Salsberg reported that a cows age and stage of lactation needs to be considered when evaluating SCC, but that it was not clear whether the factors directly affected SCC or were a result of increased rate of IMI with
increasing age and stage of lactation. (Salsberg, et al., 1984) Sobreira et al. recently examined composite SCC’s and found that the Log10SCC was not different for infected and uninfected bovines, although it was not clear whether or not the type of infection was taken into consideration. (Sobreira, 2009)

Mastitis pathogens have often been described as major or minor pathogens based on their tendency to elevate SCC. Comparing SCC in context of microbiological culture, the geometric mean of negative quarters have an SCC of 68,000 cells/mL, quarters infected with minor and major pathogens have SCC of 130,000 cells/mL and ≥ 350,000 cells/mL, respectively. (Djabri, et al., 2002, Pyorala, 2003) Specifically, the mean of SA infected quarters was 357,000 cells/mL. In contrast, Neave (1973) noted that although somatic cell counts may fluctuate, some dropping below 500,000, all infections had cell counts greater than one million at some point during sampling. (Neave, 1973) Buelow et al attempted to classify SA infected quarters using a 250,000 cell/ml cut-off resulting in 40 false positive and 1 false negative classification. (Buelow and 1996) Buelow’s findings suggested that composite SCC had low sensitivity for detecting SA IMI (31-54%). Although quarter level SCC proved more sensitive (87-95%), since quarter level values are not readily available and represent a substantial expense, such cut-offs are of questionable value to the veterinary practitioner. In examining the use of SCC in mastitis control programs, Reneau noted that there was a difference in the magnitude of increase between pathogens, but the degree of the difference was unreliable as a predictor of the pathogen causing the IMI. (Reneau, 1986) Although this published work does not negate the utility of test day information, it certainly brings to light the critical need for developing coherent and consistent standards for interpreting SCC in the context
commonly provided (composite samples) and in relation to specific pathogens being addressed. An additional consideration which should be made in using test day cell count information is that, by definition, the information is historical and unless cell counts were obtained at the same time as sample collection for culture, the reported SCC may not reflect the state of the gland at the time the sample was taken for culture.

In spite of the limited application of SCC in efficiently identifying SA IMI, avenues of exploration remain with regards to noted differences in SCC elevations amongst SA IMI. Given the increased availability and application of molecular techniques, interest in the effects of strain differences amongst SA on SCC and other aspects of pathogenicity have grown. Sol et al. reported that β-lactamase positive strains of SA had a more persistently higher SCC than other strains. (Sol, et al., 2000) Future work is necessary to clearly identify critical differences amongst strains and the potential effects such differences may have in the management of SA IMI.

### 2.3 Treatment of S. aureus

As SA remains the most prevalent contagious mastitis pathogen, it has been subject to many treatment modalities each presenting significant cost and risk to the individual operation.

Experiments have shown that the earlier an infection is treated, the higher the likelihood of microbiologic cure. (Hillerton and Berry, 2005) Thus far, cow-side detection methods have proven either impractical or inefficient in early detection of mastitis to allow for earlier treatment. It is also important to recognize that the majority
of mastitis is subclinical, resulting in an IMI that can be either short or chronic (lasting greater than 4 weeks) in duration, thus presenting another major stumbling block to early treatment. (Barlow, et al., 2009) When reviewing the literature, it is also important to keep in mind that the vast majority of studies evaluating treatment efficacy of IMI rely on trained employees to grossly detect mastitis, thereby limiting the case representation to those with clinical infections. (Deluyker, et al., 1999) Despite efforts to train those responsible for screening, there will inevitably be inconsistencies between milkers, herdspersons and veterinarians that limit broad application of many of the published findings.

Although initial perceptions of the efficacy of intramammary antibiotic were quite positive, with reported cure rates of susceptible pathogens ranging from 35% to 91%, few practitioners today would agree with such predictions. (Erskine, et al., 2003, Hoe and Ruegg, 2005) The reason for treatment failure is multifactorial including the host, pathogen, treatment, and duration of both the infection and treatment to name a few, and is therefore difficult to isolate. Variability in treatment outcomes was explored by Barlow et al. who examined the effects of lactational therapy targeting subclinical mastitis, developed a mathematical model concluding that the benefits of therapy were dependent on a complex function of cure-rates and transmission rates which have a very broad range. (Barlow, et al., 2009) The proceeding section includes discussion of the many limitations of our understanding of treatment effectiveness in SA IMI, in addition to the impact of the individual cow.
2.3.1 Defining Cure

Determining cure rates of SA IMI is fraught with as many pitfalls as diagnosis. It should come as no surprise that without a consistent definition for SA IMI, there is also no consistent definition for cure. In evaluating cure, one must consider whether the goal is a clinical outcome or microbiological. While some reports define cure as the resolution of clinical signs, others use normal SCC or negative culture results or any combination of the three. (Erskine, et al., 2003, Hoe and Ruegg, 2005) Although clinical cure is most easily evaluated, despite being subject to inter-operator variability, microbiological cure poses an even greater challenge. Review of the literature finds that definitions for cure include but are not limited to: a single negative culture; negative culture and normal appearing milk and gland, or negative culture, normal appearing milk and gland and a normal SCC. (Petitclerc, et al., 2007, Sol, et al., 2000) Exploring the impact of differences in definition, Deluyker et al. found clinical cure rates ranging from 51.8% to 62.5%, which far exceeded other reported combined cure rates (clinical and bacteriological) of 21.7%. (Deluyker, et al., 1999)

Examining SA IMI specifically, cure rates ranged from 4% to 90%. (Barkema, et al., 2006, Erskine, et al., 2003, Oliver, et al., 2004a, Sears and McCarthy, 2003, Sol, et al., 2000) When using microbiologic culture to define cure, some have evaluated cure by a single sample, others have employed duplicates at varied days post treatment. (Constable and Morin, 2002, Deluyker, et al., 1999) Cure was defined by Owens et al. as 2 negative duplicate quarter samples at parturition, Dingwell et al. required 3 negative samples, Deluyker et al. required negative samples at 21 and 28 days post treatment, Middleton required 3 consecutive negative cultures 1 month apart, while
Oliver required 2 negative samples 14 and 28 days after treatment in a lactational trial and at 7, 14 and 30 days after calving in a heifer trial. (Deluyker, et al., 2005, Dingwell, et al., 2003, Middleton and Fox, 2001, Oliver, et al., 2004a, Oliver, et al., 2004b, Owens, et al., 2001, Owens, et al., 1997) In contrast, Daley et al., studying experimental infections with Newbould strain considered quarters cured when culture negative for 14 consecutive days. (Daley, et al., 1991) Again, while parity, SCC, pathogen strain, the number of quarters infected, the number of times culture positive, shedding and treatment durations have all been cited as host-level factors affecting cure rates (Barkema, et al., 2006), it is difficult to compare studies due to variability in diagnosis, treatment and measured outcomes. (Deluyker, et al., 2005, Dingwell, et al., 2003, Dingwell, et al., 2006)

Additional considerations should be made when evaluating cure using negative culture results as they may be misleading for several reasons: The inhibition of bacteria exposed to antimicrobials may vary depending on the bacteria and the presence of abscesses within the udder parenchyma; other infections, such as those caused by gram negative bacteria, may never provide positive culture results in spite of clinically abnormal milk and high SCC for extended periods of time; and principles of exclusion have varied greatly among treatment trials. While some studies have excluded cows requiring extended therapy, or with severe symptoms, others include them, at times as treatment failures or as separate treatments all together. (Deluyker, et al., 1999) Taponen et al. excluded cows in a treatment trial for clinical mastitis that had persistent (months) elevated cell counts or infections that remained through the dry period. (Taponen, et al., 2003) However, although they reported increased cure rates in first lactation cows, there was no explanation offered as to how they could be sure a subclinical infection was not
present in prior lactations. In addition, some infections were experimental with strains now believed to have low udder affinity, making the application of results questionable.

2.3.2 Treatment and the Utility of Antibiotic Profiling

Examining treatment failures, many have explored the utility of identifying the pathogens prior to treatment and evaluating antibiotic sensitivity profiles. Although it is important to consider the basic differences between the major pathogens when evaluating treatment, it is rare that preliminary treatment decisions are made in consideration of individual culture results or antimicrobial sensitivity reports. A more pragmatic approach suggested is to monitor isolates so that herd trends may be identified and used to guide therapy. (Owens and Watts, 1988) However, even a pragmatic approach may prove problematic as little correlation has been found between treatment outcome and the susceptibility patterns of tested bacteria. (Constable and Morin, 2002, Constable and Morin, 2003, Hoe and Ruegg, 2005) In review of the utility of results of Kirby-Bauer antimicrobial sensitivity tests, Constable et al. determined that results were of no value in predicting either the duration of clinical signs or bacterial cure rates in clinical mastitis cases. (Constable and Morin, 2002) The single exception in his study was in the treatment of mild clinical mastitis caused by gram-positive organisms (excluding SA) with intramammary cephapirin. In such cases, Constable et al. (2002) found that susceptible bacteria experience a significantly higher bacteriologic cure rate than resistant isolates 28 days post treatment. The reasons for this lack of translation from the lab-bench to the milking-parlor are many, but one primary consideration must be noted: None of the approved standards for veterinary pathogens has been validated for mastitis pathogens.
Sensitivity screening such as the Kirby-Bauer, designed to mirror the concentration of antimicrobials in serum and interstitial fluid, are fundamentally flawed when applied to mastitis pathogens. (Constable and Morin, 2002) Without an adequate database of in vitro MIC values for clinical mastitis pathogens, the majority of breakpoints used are derived from human or non-bovine species, leaving us to presume similar effectiveness. When evaluating the efficacy of antimicrobials based on MIC or disc diffusion we must also recognize that the vast majority of breakpoints established are not based on clinical cases of bovine mastitis, rather they are in clinically normal and healthy animals. (Constable and Morin, 2003) Results are often extrapolated from similar bacterial species isolated from different diseases such as bovine pneumonia and sometimes in different animals, such as pigs or humans, presuming equivalent serum concentrations can be reached while knowing little of how such concentrations translate to milk. (Hoe and Ruegg, 2005) In fact, there is conflicting data as to whether or not the in-vitro results of bacterial inhibition tests translate well into the udder in-vivo. A reflection of this obstacle was shown by Owens who noted that during a treatment trial, several quarters continued to shed SA while being treated in spite of adequate levels of antibiotics. (Owens, et al., 1988) Currently in-vitro information is sparse and based on subclinical isolates. Only three antimicrobials have predicted concentrations based on their intended target, the mammary gland: Pirlimycin, penicillin-novobiocin and ceftiofur hydrochloride. (Constable and Morin, 2003) However, it should be noted that the breakpoints for Pirlimycin and penicillin-novobiocin were established in healthy mammary glands and are lacking field efficacy data. (Constable and Morin, 2003) In addition, the NCCLA uses representative antibiotics for each of the given classes of antibiotics for susceptibility
testing, which often do not contain the same active compounds found in the commercial preparations. Therefore, we should not be surprised that in-vitro tests fail to meet our expectations when applied to the infected mammary gland. (Constable and Morin, 2003)

Systemic therapy has been suggested as an appropriate route of administration for chronic IMI such as SA. However, drugs meeting the pharmacokinetic requirement for adequate tissue distribution to the udder are limited to macrolides, trimethoprim, tetracyclines and fluoroquinolones. As extra-label options are limited, practical applications of antibiotics are restricted to the udder compartment, focusing on the ducts and alveoli of the mammary gland. (Erskine, et al., 2003) Currently there are three mastitis formulations that have been validated against bovine mastitis agents and they are: pirlimycin, penicillin-novobiocin, and ceftiofur hydrochloride.

The most common motivation for extra-label drug use in dairy cattle is in the treatment of IMI. (Smith, et al., 2005b) Although novel combinations (including compounding) of antibiotics have been examined, both locally and parenterally, few would currently pass muster under AMDUCA, thus their application is again limited. Such extra-label use of antibiotics present extreme risk with regard to violative residues and FARAD recognizes that combination therapies make withdrawal recommendations difficult to assess due to limited data (Smith, et al., 2005b). As with standard therapies, reports are inconsistent as to whether or not extended off-label therapies provide for increased treatment success and are therefore expensive and often viewed as impractical. (Hoe and Ruegg, 2005)

While results of in-vitro susceptibility tests against environmental mastitis pathogens have met with mixed results, some have reported that such tests have proven
useful in predicting the potential for cure with SA IMI specifically. (Makovec and Ruegg, 2003) Several reports have been made available regarding the susceptibility of SA from bovine mastitis isolates. Before such reports are examined, it is important to consider the limited application of such data. A meta analysis evaluating treatment of subclinical SA mastitis found that the MIC test was of no use in predicting bacteriologic cure. (Constable and Morin, 2003) Additionally, Owens reported in a study examining 671 staph species isolates from 7 dairy herds, that penicillin resistance in SA ranged from 0 to 60% (7% average), while non-aureus species exhibited a greater percentage of resistance. (Owens and Watts, 1988) Such farm dependent variation in susceptibility limits broad application of published sensitivities when making treatment decisions. Sears et al. recently proposed a method of selecting an antibiotic and dose which included selecting 10 isolates from a herd for testing and using a dose which exceed the MIC for 9 of the 10 isolates (MIC90). (Sears and McCarthy, 2003) Whether such doses are allowable under AMDUCA or available in approved products was not discussed and present a major obstacle in applying the practice.

It has been noted that SA often survives therapy, either being sequestered within abscesses in the udder parenchyma or by the induction of L-forms which lack a cell wall, making them difficult to culture. (Erskine, et al., 2003) Ownes (1988) demonstrated that strains obtained from bovine IMI, when exposed to β-lactams and a combination of penicillin and streptomycin antibiotics, were induced to L-forms. (Owens, 1988) However, while some strains were more likely to convert to L-forms, Owens (1988) found no relationship between resistance to antibiotics and the development of L-forms.
thus the relevance of the development of L-forms remains unclear with regard to treatment failure. Work has recently been completed examining the effects of biofilm formation on antimicrobial resistance. Rather than the traditional MIC, an extended biofilm antimicrobial assay was used to examine 78 SA strains and discriminated between strains eradicated at concentrations below CLSI breakpoints and those that were not. (Lam, 2008)

2.3.3 Stage of Lactation and its impact on Treatment

2.3.3.1 The Dry Period

Targeting the application of antibiotics prior to dry-off has been perceived as the most economical method of curing subclinical IMI in addition to decreasing the risk of violative residues. This pragmatic approach recognizes that, the ability to use higher concentrations of antibiotics at this time, in addition to a the benefits of increased retention time within the mammary gland, provides better cure rates. (Nickerson, et al., 1999) Blanket dry cow therapy, treating every quarter of every cow with an intramammary tube at the end of lactation, has been standard practice in the United states for over 30 years. (Erskine, et al., 2003) Targeting subclinical infections that have persisted through the lactation and preventing new infections during the dry period, dry cow therapy has provided variable efficacy in curing SA, cure rates ranging from 40-70%. (Sears and McCarthy, 2003) Although the majority of dry tubes target bacteria such as SA, other studies report cure rates for SA during the dry period from 20-80%. (Dingwell, et al., 2006, Nickerson, et al., 1999) In a study evaluating the efficacy of intramammary Tilmicosin at dry-off in preventing new infections, Dingwell observed
that 10.7% of new IMM were due to SA; however, the question remains as to whether they were truly negative prior to dry off due to a lack of a standard case definition.(Dingwell, et al., 2002)

One common fault amongst all studies conducted, was not identifying cows that had previously undergone DCT. Studies have reported that multiparous cows experience lower cure rates with dry cow therapy.(Deluyker, et al., 2005, Sol, et al., 1994) This should not be surprising considering that the vast majority of cows would have seen at least one administration of DCT prior to inclusion in the study, thereby eliminating the majority of infections amenable to treatment.

2.3.3.2 Lactational Therapy

After review of the literature and considering the pharmacodynamics of treatment approaches, Erskine concluded that if standard therapy proved ineffective, the best approach was to provide extended therapy rather than switching drugs or increasing doses.(Erskine, et al., 2003) Others have also examined extended therapies in both clinical and subclinical cases and have had variable outcomes, reporting cure rates from less than 20% to greater than 80% depending on the drug and length of treatment.(Deluyker, et al., 2005, Sears and McCarthy, 2003, Sol, et al., 2000) Deluyker compared subclinical cases treated with pirlimycin and found cure rates significantly higher with extended therapy (86%, 8days) when compared to standard therapy (56%, 2 days) and control (6%, no treatments).(Deluyker, et al., 2005) Sol et al. found that cure rates in clinical cases were lower for β-lactamase positive isolates and higher in cows
with lower SCC (1 Ln) on the previous test day in first and second lactation with β-
lactamase negative infections. (Sol, et al., 2000) Oliver found that extended lactational
therapy of subclinical SA with ceftiofur for 8 days provided significantly better cure rates
than those quarters treated for 2 and 5 days, cure being evaluated at 14 and 28 days after
treatment. (Oliver, et al., 2004a) Again, when comparing treatment studies, is it important
to consider case definitions, as little consistency is present in the definition of infection or
cure, treatment outcomes of clinical cases are very difficult to compare.

2.3.3.3 Pre-partum Treatment of Heifers

The NMC reports that SA is a contagious mastitis pathogen, the majority of
infections being spread from cow to cow during milking. (NMC, 2004) Such a
perspective misrepresents the significant impact SA mastitis has on the future of a dairy
herd. It has been demonstrated that dairy heifers are at risk of developing SA IMI prior to
exposure to either a milking unit or milking parlor. (Oliver, et al., 2004b, Owens, et al.,
2001) Owens found an average of 15.4% of heifer quarters were infected with SA and
Oliver reported an SA IMI prevalence of 8% in Jersey heifers and 30% in Holstein heifers
14 days prior to calving. (Oliver, et al., 2004b, Owens, et al., 2001) Infections with SA
have been diagnosed as early as 9 months of age. These infections present prior to
freshening might, in fact, be chronic and refractory to treatment. Several studies have
been conducted examining the pre and post-partum treatment of SA infections in first
lactation cows. An advantage of this approach is that it decreases the number of animals
treated during lactation, prevents wastage of milk and the removes the risk of violative
residues (provided heifers are treated 60-45 days prior to parturition to allow for appropriate with-hold times). Owens et al. found heifer cure rates were higher for pre-partum treatment (90%) than for lactational therapy (50%) and that there was no difference in the antibiotic used or the time of administration. (Owens, et al., 2001) Interestingly, of the heifers not treated, only 8% remained positive at parturition. Of the treated group, 5% remained positive at parturition. Although pre-partum treatment is indeed effective at eliminating a large proportion of all IMI, Borm reported that such strategies may be ineffective at significantly lowering SCC or increasing milk production in first lactation heifers. (Borm, et al., 2006) However, when considering pathogen type, Borm did find that heifers not cured with a major pathogen did have higher SCC than those that cured with either minor or major pathogen (lower SCC was associated with treatment NOT cure).

Yet many questions remain. What defines a cured quarter? Is a clinically normal quarter sufficient? Such questions are critical, given that the majority of SA IMI infections are subclinical, thus negating the need for treatment in the vast majority of SA IMI. Is a “normal SCC” all that is required although bacteria may still persist? What are the implications of a clinically normal quarter with a SA infection and a “low” SCC? These are critical questions that have yet to be answered.

2.4 Strain Differences in S. aureus

Although the negative impacts of SA have been well established, relatively little is understood about the molecular basis for the infectivity and pathogenicity of various SA strains. Studies examining the molecular epidemiology of SA suggest that there are
genetic subsets of strains well adapted to the udder. (Herron-Olson, et al., 2007) Attempts to consolidate isolates from several source across the globe suggest that relatively few clones are responsible for the majority of IMI’s. (Smith, et al., 2005a) Work completed by Smith et al. revealed that the majority (87.4%) of isolates were distributed within a single clonal complex and that subtypes of this complex were adapted to the mammary gland and the bovine environment, thus allowing for a global distribution. (Smith, et al., 2005a) Recent data by Alves et al. comparing bovine, ovine and human isolates, suggests that the adaptability of SA is a host specific genetic trait rather than tissue specific genetic trait. (Alves, et al., 2009) Developing a better understanding of such strain specialization may be the key to developing better management and control strategies for SA IMI.

2.4.1 Interpretation of PFGE

To effectively study the relatedness amongst bacterial strains, a reliable technique to accurately differentiate strains is required. PFGE has been established as an accepted and powerful method of discriminating amongst SA isolates with one notable limitation: between-lab reproducibility. One of the admitted limitations in the application of PFGE is the apparent lack of consistency in analyzing the fragment patterns, allowing different conclusions to be drawn from the same data. (Tenover, et al., 1995) Therefore, comparisons between studies are difficult as interpretations of PFGE results between labs are subjective and inclusion of isolates is variable. While some studies have limited strain examinations to clinical isolates, others have only included strains in the analysis
which have at least three representative isolates, possibly diminishing the apparent
diversity of the SA population within and amongst herds. (Sabour, et al., 2004)

Tenover et al. published a set of guidelines for interpreting DNA restriction
patterns generated by PFGE for small sets of isolates (≤ 30) in examining outbreak
occurrences when more expensive digitized programs are not readily available. (Tenover,
et al., 1995) The scheme presented by Tenover et al. requires that each isolate be
represented by at least 10 distinct PFGE fragments. Tenover et al. proposed that isolates
with fewer than 50% fragments in common are considered unrelated types, while patterns
differing by at most 3 are considered sub-types of the epidemiologic strain of interest.
Using these criteria, strains may be classified as, indistinguishable, closely related (differ
by a single genetic event resulting in 2-3 band difference), possibly related (differ by two
genetic events with 4-6 band differences) or unrelated (seven or more band differences).
However, Tenover stated that these guidelines were meant for outbreak investigations
over very short periods of time and not necessarily appropriate for large populations over
extended periods. A similar interpretation was put forth by Zadoks et al., where a four
band difference between macrorestriction patterns was interpreted as a different
pulsotype while 3 or fewer differences were considered subtypes. (Zadoks, et al., 2002)
Additionally, Sabour et al. further specified using a band position tolerance of 0.4% when
creating dendrograms using commercial software. (Sabour, et al., 2004)

2.4.2 Strain Diversity of S. aureus

Several reports describing the molecular diversity of SA within farms have been
published. Regardless of method, the majority have found that SA IMI isolates from a
single farm represent clonal populations revealing a predominant strain type with little strain diversity. (Anderson, et al., 2006) Zadoks et al. reported that PFGE could effectively be used in the characterization of SA isolates and found that subclinical SA infections with low SCC were associated with a specific pulsotype. (Zadoks, et al., 2000) Zadoks et al. (2000) also noted that since only a few predominant types were identified, the contagious nature of SA was substantiated. Additional support that a limited number of bovine SA strains are responsible for the majority of mastitis cases has been published. (Sabour, et al., 2004) Comparisons between human and bovine strains have also illustrated a reasonable degree of host specificity and that although possible, transfer of SA between human and cattle is rare. (Gilot and van Leeuwen, 2004, Zadoks, et al., 2000) Efforts attempting to describe epidemiologic relatedness of SA amongst mastitis isolates within and between herds continue. Such studies have identified that there are strains and distributions of strains that predominate at the herd and geographic level. (Sabour, et al., 2004)

Zschock et al. examined the similarity of PFGE patterns from 26 SA isolates from 16 cows on a single dairy. (Zschock, et al., 2000) Zschock et al. reported that all strains had the same antibiotic resistance pattern (resistant to penicillin-G and ampicillin), and 24 of the 26 isolates were represented by one restriction pattern, suggesting that a single strain was responsible for the majority of SA IMI within the herd. (Zschock, et al., 2000) A second report by Capurro et al. also describes PFGE patterns, this time amongst 5 dairies, reporting that each herd had a single pulsotype that predominated. (Capurro, 2009) However, a consideration must be noted. The study included dairies with “mastitis problems”. Although it is unclear what constitutes a “problem”, selecting such
herds may bias the strain representation toward isolates that are more contagious, thus explaining the associated pulsotype predominance. In contrast, a study examining 8 separate herds found that although 75% of the herds did have a predominant strain accounting for 53% to 100% of the isolates (n=43), the number of strains in each herd ranged from 1 to 13 with 88% of the strains determined to be unique to each individual herd. (Middleton, et al., 2002a) Middleton et al. (2002b) also examined the diversity of SA in herds dependent on imports for either replacements or expansion. The study revealed that herds purchasing replacement heifers had a greater prevalence of SA IMI than herds purchasing lactating cattle. In addition, PFGE strain typing revealed 82% of the strain types were unique to one herd and that herds purchasing replacement heifers had more strains than closed herds. (Middleton, et al., 2002b) Haveri et al. reported similar findings in 2008, comparing a closed herd with 3 pulsotypes to a herd using off site heifer growers with 7 pulsotypes. (Haveri, et al., 2008)

2.4.2.1 Difference in Pathogenicity amongst Strains

Historically, differences seen in rates of transmission from farm to farm have been attributed to differences in management and milking practices. However, application of modern technology may prove otherwise. Middelton et al. used PFGE typing to explore differences in pathogenicity between SA strains. In the published study, there appeared to be no significant difference between SA strain and effects on SCC or NAGase activity. (Middleton, et al., 2002a) It was concluded from this study that differences in the outcome of infection were due in large part to cow-to-cow variation. Haveri et al. also employed PFGE to examine the pathogenicity of SA IMI, subjecting
134 sub-clinical (28) and clinical (75) isolates to comparison. In contrast to Middleton, Haveri et al. found an association between strain types, the severity of infection, and the persistence of infection following treatment. (Haveri, et al., 2005) In addition, LeThanh et al. published findings implicating the type of hemolysis in the severity of mastitis, associating beta or weak beta hemolysis with sub-clinical rather than clinical infections. (Lam, 2008) Whether such phenotypic differences are indeed related to virulence factors have yet to be proven, but are of great interest in developing management and control strategies.

2.4.2.2 The Effect of S. aureus Strain on MIC and Treatment Outcome

Antimicrobial resistance is often presumed to be one of the major reasons for treatment failure. However, with the exception of penicillin, the literature demonstrates that the majority of SA isolates are indeed susceptible to the antimicrobials found in approved treatment formulations. Examining whether there were strain specific patterns in antimicrobial sensitivity, Anderson found that although resistant isolates were only recovered from 3 of 24 farms (86% of the isolates were pansusceptible), PFGE clusters exhibited good correlation with susceptibilities. (Anderson, et al., 2006) An important consideration in evaluating this work, however, includes the fact that only isolates with \( \geq 5 \) colonies were included and all of the isolates were likely clinical (although not specified) as they were submitted to a diagnostic lab from 2 states and also may have been subject to previous treatment.

Sabour et al. examined 212 SA isolates from 58 farms, reporting that a total of 24.5% of the isolates were resistant to at least one antimicrobial, resistance to penicillin
being the most common (9.9%), followed by sulfadimethoxine. (Sabour, et al., 2004) While Sabour’s et al. (2004) findings agree with Anderson et al. who reported 12%, they are very different from Erskine who has reported resistance to penicillin in 46% of Michigan dairy herds and Makovec et al. who reported resistance in 35.4% of Wisconsin herds. (Anderson, et al., 2006, Erskine, et al., 2002, Makovec and Ruegg, 2003) Sabour et al. (2004) also reported no difference in the level of resistance before and after antibiotic therapy administered at dry-off.

Cure rates for SA IMI using antibiotics over the dry period have varied from 20%-80%, while self-cure rates as high as 38% have been reported. (Dingwell, et al., 2006) In a study examining cure rates following DCT, Dingwell subjected 121 SA isolates from 40 herds to PFGE and identified 3 lineage groups (A,D and F). (Dingwell, et al., 2006) Overall, 88% of the herds had strains confined to a single lineage, although a high degree of heterogeneity was noted. This is similar to what others have reported and is consistent with the contagious nature of SA. Examining each group, Dingwell found that lineage group had a significant impact on the probability of cure of subclinical IMI (cows infected with strains within lineage group D treat with benzathine cloxacin had higher cure rates in spite of higher SCC at dry of than groups A or F). (Dingwell, et al., 2006) Such results increase interest in further developing systems by which we may use strain typing technology to better manage treatment and culling decisions of SA cows. The ability to identify strains more likely to cure will allow for selective treatment and culling, thereby saving producers the cost of both wasted treatment and unnecessary replacements.
2.4.2.3 The Impact of the Application of Newbould Strain of S. aureus in Experimental Intramammary Infections

Several studies evaluating both shedding and cure rates have used experimental infections of the Newbold 305 strain of SA (ATCC 27940). (Middleton, et al., 2006, Nickerson, et al., 1999, Owens, et al., 1988) Recent studies employing multilocus sequence typing have revealed that the Newbould strain of SA, commonly used in experimental SA IMI evaluating both shedding and treatment, appears to have low affinity for the udder and is more likely to colonize the teat skin. (Barkema, et al., 2006, Smith, et al., 2005a) Therefore, results based on such experimental infections should be interpreted with caution. The impact of this finding is substantial in that much of what we currently think about the epidemiology of SA is, in fact, based on experimental infections. Whether or not our understanding truly reflects that of natural infections will only be known once extensive studies of naturally occurring infections are completed in conjunction with standardization of both methodology and definitions of infection and cure.

2.5 Motivation for research

Veterinarians occupy a very unique niche in that they accept as their professional duty a unique obligation that is founded in both the humanity of man and the humanity provided to animals in our care. As a veterinary practitioner, my primary motivation has been, and continues to be, the health and well being of dairy cattle. Clearly mastitis, particularly that caused by SA, has a significant impact on the welfare of dairy cattle, in addition to its profound impact on the success of individual farms and on food safety.
My goal is to provide information that helps practitioners better understand SA so that we may develop better prevention and management strategies protecting the health of livestock. Identifying cows with either a higher probability for cure or strains more virulent and prone to dissemination within the herds may prove useful in minimizing the losses due to SA. Reviewing the literature, it is clear that significant gaps in our understanding of the epidemiology of SA IMI remain. These gaps remain in part due to a lack of continuity within the scientific community and in part due to new evidence revealed through the application of modern molecular techniques in examining pathogens. To begin to fill these gaps, standards must be set and extensive, yet basic, information must be gathered so that we may make progress towards understanding the true epidemiology of SA IMI.
2.6 Endnotes

a. Neave’s criteria for diagnosis of an IMI via microbiological methods:

1. That mastitis of economic importance is generally of bacterial origin and mainly caused by bacteria that are readily isolated by relatively simple methods.
2. The most common primary pathogens are normally secreted in sufficient numbers from sub-clinically affected quarters to be detected when a small volume of foremilk is examined.
3. The pathogens are regularly secreted from infected quarters in detectable numbers so that it is unusual to fail to recover the pathogen either from one or from two or more successive foremilk samples.
4. The infections detected usually produce a marked increase (> 10 fold) in somatic cells in the milk of the affected quarter.
5. The pathogens are infrequently found in milk from uninfected glands if samples are taken aseptically or are rarely found in two or more successive samples about a week apart.
2.7 References


Buelow, K. L. T., Chester B.; Goodger, William J.; Nordlund, Kenneth V.; Collins, Michael T. and 1996. Effect of milk sample collection strategy on the sensitivity and specificity of bacteriologic culture and somatic cell count for detection of


USDA. 2008. Milk Production, Disposition and Income 2007 Summary. USDA.


CHAPTER 3

THE EFFECTS OF INOCULUM VOLUME ON THE MICROBIOLOGICAL DETECTION OF NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS

3.1 Introduction

The most prevalent contagious mastitis pathogen in the United States is *Staphylococcus aureus* (SA). In 2007, SA was detected in 43% of bulk tanks examined. (USDA, 2008) Control programs targeting SA are varied and have been inconsistently applied throughout the dairy industry with equally variable success. (Fox & Hancock, 1989; Nagahata et al., 2007; Zadoks, Allore, Hagenaars, Barkema, & Schukken, 2002) Although there is no single explanation for the inconsistencies, the cost and perceived inaccuracy of diagnosis are acknowledged challenges in the broad application of effective control programs. The success of any mastitis control program lies in the ability to detect subclinical intramammary infections (IMI). Often present as a subclinical infection, the control of SA is contingent on accurate diagnosis of IMI, yet there remains disagreement on what constitutes a definitive microbiological diagnosis of a SA IMI. As evidenced in a meta-analysis published in 2002, there has been little consistency in defining either the inoculum volume, number of samples or colony forming units (cfu) required to define any type of IMI. (Djabri, Bareille, Beaudeau, & Seegers, 2002) However, Torres et al. (2009) recently published data proposing
standard microbiological definitions of IMI caused by major and minor pathogens based on single quarter milk samples. (Torres, Rajala-Schultz, & DeGraves, 2009) In addition to the current lack of standardized definitions of IMI, much of the information used to describe SA IMI has been collected from experimental IMIs. (Buelow, 1996; Daley et al., 1991; Middleton et al., 2006; Sears, Smith, English, Herer, & Gonzalez, 1990) Recent investigations examining the molecular relatedness of experimental and naturally occurring strains of SA (Smith et al., 2005) have challenged the conclusions from previous studies based on experimental infections. The goal of this study was to examine the effect of inoculum volume of milk samples on the detection of naturally occurring SA infections so that a standard inoculum can be established, thus providing better comparability between studies examining SA IMI in the future.

3.2 Materials and Methods

Microbiological results were combined from 2 separate field studies examining the epidemiology of naturally occurring SA IMI using 2 different sampling schemes, a daily sampling scheme over 63 days and a weekly sampling scheme over the cow’s entire lactation. From the study employing daily sampling, data was used from 9 SA positive quarters (7 SA positive cows) that were sampled daily during 3 separate sample periods, each lasting 21 days. One quarter, negative for SA from each SA positive cow was also sampled in addition to 7 additional quarters from SA negative cows (14 total). From the study using weekly sampling, culture results from 34 SA positive quarters (25 SA positive cows) collected during the first 10 weeks of each cow’s lactation were used. Samples were also collected from 22 quarters, negative for SA in SA positive cows in addition to 5
SA negative quarters from 3 SA negative cows. Approximately 12 mL of foremilk was collected from each quarter for culture according to NMC guidelines. Milk samples were kept frozen and thawed at room temperature for microbiological culture and immediately frozen again, kept at -80°C until cultured a second time. The initial culture procedure employed standard NMC (2004) guidelines, applying 0.01mL of milk on TSA blood agar plates (5% sheep blood, Remel, Lenexa, KS) using disposable, calibrated loops. Plates were incubated at 37°C and screened at 24 and 48 hours according to NMC (2004) guidelines. A diagnosis of SA was made based on colony morphology, positive catalase test, presence of hemolysis and a positive coagulase test. A SA IMI was defined as a quarter having at least 1 of the first 3 samples (daily or weekly) culture positive with ≥1cfu of SA/0.01mL. Individual quarter samples with ≥1 cfu of SA were considered positive. Samples considered contaminated (3 or more apparent bacterial species) were excluded from the analysis (n=25). Milk samples that were culture negative for SA using a 0.01 mL inoculum from SA IMI quarters and all samples from non-SA quarters were thawed a second time and cultured using 0.1mL of milk. Sensitivities and specificities of a single microbiological culture in identifying SA IMI were calculated considering each quarter sample independently (roctab, STATA v.10). Quarters with no history of SA, but repeatedly testing positive for SA at some point after the third sample, were considered new infections and not included in the analysis (n=3). Quarters culture positive for SA in cows that were treated with intramammary or systemic antibiotics at anytime during the current lactation were not included in the analysis. Culture results from quarters that ceased lactating prematurely and cows that
were culled due to illness (other than mastitis) during the study were included provided there was no evidence of antibiotic treatment during the sample period.

### 3.3 Results

#### 3.3.1 0.01mL Inoculum

A total of 1,583 milk samples were included in the analysis and the results of using a 0.01mL inoculum are presented in Table 1. Considering a quarter culture positive at ≥1 cfu, using a 0.01mL inoculum, there were 9 culture negative samples from known SA positive quarters (2.0% false negatives) and no culture positive samples from known negative quarters (false positives) from the sample collected daily. The sensitivity and specificity of microbiologic culture examining daily samples is 97.7% and 100% respectively. In contrast, to the daily samples there were 56 culture negative samples from known SA positive quarters (17% false negatives) and 5 culture positive samples from known SA negative quarters (2% false positives) from the samples collected weekly resulting in a sensitivity of only 82.9% and a specificity of 98.0%. Of the 56 false negative samples, 40 (71%) were from 8 of the 34 SA positive quarters. Combining the results of both studies, the 0.01mL inoculum results in a sensitivity of 91% and a specificity of 99.4%.

#### 3.3.2 0.1 mL Inoculum

The results of using a 0.1mL inoculum are presented in Table 2. Considering a quarter culture positive at ≥1 cfu, using a 0.1mL inoculum, there was only 1 culture negative sample from known SA positive quarters (0.2% false negatives) and no culture
positive samples from known negative quarters (false positives) from the sample collected daily. The sensitivity and specificity of microbiologic culture examining daily samples was 99.8% and 100% respectively. Examining the weekly samples there were 22 culture negative samples from known SA positive quarters (6% false negatives) and 6 culture positive samples from known SA negative quarters (2% false positives) resulting in a sensitivity of only 93.8% and a specificity of 97.6%. Of the 22 false negative samples collected in the weekly samples, 17 (77%) were represented by 6 of the SA positive quarters. Combining the results of both studies, using a 0.1mL inoculum resulted in a sensitivity of 96.8% and a specificity of 99.3%. The cfu count on false positive quarters ranged from 1 to 8 cfu/0.01ml. The number of cfu/0.1mL in false positive samples ranged from 2-95. All samples that were SA culture positive from SA negative quarters (n=6) were from 5 SA positive cows. The single SA negative quarter that cultured positive twice was in a cow with 2 SA positive quarters and it was culture positive using both the smaller and larger inoculum. There were no false positive cultures from SA negative cows.

3.4 Discussion

Great variation in diagnostic schemes prevail throughout mastitis research as there is little agreement on which methods and interpretation of microbiological culture should be followed in defining a gland as being infected. The widely accepted standard inoculum volume for mastitis diagnostics is 0.01 mL; however, there remains great inconsistency in what has been used and reported in research. While some have used inoculums of 0.01mL, others have increased inoculum volumes to 0.05mL and 0.1mL,
thereby increasing the sensitivity of microbiologic screening. (Deluyker, Van Oye, & Boucher, 2005; Middleton, Fox, Gay, Tyler, & Besser, 2002; Middleton, Fox, & Smith, 2001) Still, others have reported variable inoculums within a single study using 0.1 and 0.05 (Sears, Smith, English, Herer, & Gonzalez, 1990), 0.01 to 0.03 mL (Middleton et al., 2006). Buelow et al. (1996) reported that an inoculum volume of 0.1mL had the best predicted sensitivity for detecting SA. (Buelow, 1996) However, Buelow’s study (1996) examined experimentally infected quarters and used predictive models that assumed milk samples from SA infected quarters contain SA bacteria intermittently. In the present study examining naturally infected quarters over an extended period of time, the benefit of using a larger inoculum was clear only when considering the weekly samples and after combining the results of the two studies. Considering the daily samples alone, there was only a 2% difference in the sensitivity of culture using the larger inoculum. However, it is important to consider that the daily study consisted of only 7 cows on a single dairy, which may have limited strain representation. Using the smaller inoculum, SA was detected in 98% of all the daily samples collected from SA positive quarters. In contrast, the study using weekly samples examined milk from 25 cows on 2 different dairies and may therefore reflect a greater diversity in strain types. Using the smaller inoculum, weekly samples detected SA in only 83% of the samples from positive SA quarters. Of the 56 total negative samples from SA positive quarters, 71% were from 8 of the 34 known SA positive quarters. The use of the larger inoculum increased the sensitivity of culture in the weekly samples by 10.9%. Combining the findings of both studies resulted in an overall sensitivity of 96.8, nearly 10% higher than that predicted by
Buelow et al. (1996), with the number of false negative samples decreasing from 65 (9.0%) to 23 (3.2%).

The Food and Drug Administration’s guidelines in the US, published for the collection of efficacy data of mastitis treatments, require recovery of the same organism from at least 2 milk samples collected separately, 5 days apart, after treatment.(USDA, 1996) There are, however, no requirements for the diagnosis of mastitis prior to treatment. A meta-analysis by Djabri et al. (2002) clearly illustrates that variable definitions have been used throughout the literature, revealing 14 different definitions of infection based on cfu and sample frequency.(Djabri, Bareille, Beaudeau, & Seegers, 2002; USDA, 1996) The necessity of repeated (consecutive or duplicate) samples is therefore unclear and at times presents a significant economical hurdle in the diagnosis of SA IMI in management strategies. Current conventional definitions for an IMI commonly used in published literature include: The presence of the same bacteria in 2 out of 3 consecutive cultures (samples collected on different days) and the presence of the same species of bacteria in duplicate samples (2 samples collected on the same day, one after the other during the same milking). However, there have been numerous definitions employed with competing definitions used even within studies.(Borm et al., 2006; Dingwell et al., 2003; Erskine, Wagner, & DeGraves, 2003) Erskine and Eberhart reported that percentage agreement between duplicate samples was highest for contagious pathogens such as SA (94.2%), suggesting that a single quarter sample might be adequate in determining infection status.(Erskine & Eberhart, 1988) Recently, Torres et al. (2009) examined the utility of colony count in assessing IMI status comparing duplicate samples and reported that IMI could accurately be diagnosed with a single sample when cfu was
considered, and that the required number of cfu for a positive diagnosis was dependent on the type of pathogen identified. (Torres, Rajala-Schultz, & DeGraves, 2009) In that study, Torres et al. (2009) established the utility of a single sample positive with at least 1 cfu of SA/0.01mL in identifying SA IMI. That study, however, examined samples collected at a single point in the lactation (dry-off) and therefore does not reflect the potential impact of false negatives due to quarters that shed fewer numbers of SA bacteria. The current study, examining naturally occurring infections repeatedly over time, however, supports Torres’ conclusion that a single colony is sufficient to diagnose a SA IMI in single quarter sample in addition to demonstrating the benefit of using larger inoculum volumes in detecting naturally occurring SA IMI.

Much focus has been placed on our ability to correctly identify cows infected with SA. An equally important question when considering control and prevention is our ability to correctly identify cows not infected with SA. In the present study, every false positive sample was from a cow with a known SA positive quarter and there were no false positive samples obtained from completely negative cows. In addition, the 6 false positive samples obtained using the larger inoculum in the weekly sampling were from 5 different quarters. Based on our findings in naturally infected cows, SA IMI can be most accurately diagnosed using a standard 0.1mL inoculum in research and diagnostic settings and that a single quarter sample that is culture negative is sufficient to rule out the presence of SA IMI. Meanwhile, a similar sample culture positive with ≥1 CFU/0.1mL should be considered positive. Applying this standard provides a practical and economical approach to SA IMI diagnostics for research as well as practice settings.

73
There are limitations that should be noted in the data collected in the present study. This study was conducted on 2 dairies and therefore the results may be reflective of several or only a few predominant SA strains specific to these dairies. It was evident from this study that there were many more false negative samples in the weekly sampling scheme when compared to daily sampling. Given the fact that over 77% of these false negative weekly samples were represented by only six of the 34 SA positive quarters (using 0.1mL), the necessity of examining the effects of strain variation and cow to cow variation on diagnostics is clear. Quarters like the 6 noted in our study, at times shedding very low numbers of SA, or shedding at undetectable levels, are of great interest in further investigations. Repeated samples of individual quarters were also considered independently. Despite these limitations much can be gained from this examination of naturally occurring SA infections over such an extended sample period.

As far back as 1973 a call was made for science to reach an agreement as to what actually defines “mastitis” in terms of cell count or culture. (Neave, 1973) Neave pointed out then that the definitions of IMI were “not suitable” and “cumbersome” and suggested that the definition of “a normal quarter” at the time was too rigid. Because such an agreement has yet to be reached, little if any continuity or comparability between published studies is possible. This report examined naturally occurring SA IMI for an extended period with the most comprehensive sampling scheme documented to date. Future studies examining naturally infected quarters over longer periods in addition to examining the genetic relatedness of strains and shedding patterns may further aid in developing a more coherent definition for SA infections.
3.5 References


USDA. 2008. Milk Production, Disposition and Income 2007 Summary: USDA.

Table 1. Number of quarter samples that cultured positive ($\geq 1$ cfu/mL) or negative from quarters with and without SA IMI* and associated Sensitivity and Specificity of microbiologic culture, using daily samples, weekly samples and the combined results of both studies using a 0.01mL inoculum.

<table>
<thead>
<tr>
<th>Sample Scheme</th>
<th>SA + Quarters</th>
<th>SA – Quarters</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive samples</td>
<td>Negative samples</td>
<td>Positive samples</td>
<td>Negative samples</td>
</tr>
<tr>
<td>Daily</td>
<td>388</td>
<td>9</td>
<td>0</td>
<td>607</td>
</tr>
<tr>
<td></td>
<td>(n=397)</td>
<td></td>
<td></td>
<td>(n=607)</td>
</tr>
<tr>
<td>Weekly</td>
<td>271</td>
<td>56</td>
<td>5</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>(n=327)</td>
<td></td>
<td></td>
<td>(n=252)</td>
</tr>
<tr>
<td>Combined</td>
<td>659</td>
<td>65</td>
<td>5</td>
<td>854</td>
</tr>
<tr>
<td></td>
<td>(n=724)</td>
<td></td>
<td></td>
<td>(n=859)</td>
</tr>
</tbody>
</table>

* A SA IMI was defined as a quarter having at least 1 of the first 3 samples culture positive with $\geq 1$ cfu of SA/0.1mL. Individual quarter samples with $\geq 1$ cfu of SA were considered positive.

Table 2. Number of quarter samples that cultured positive ($\geq 1$ cfu/mL) or negative from quarters with and without SA IMI† and associated Sensitivity and Specificity of microbiologic culture, using daily samples, weekly samples and the combined results of both studies using a 0.1mL inoculum.

<table>
<thead>
<tr>
<th>Sample Scheme</th>
<th>SA + Quarters</th>
<th>SA – Quarters</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive samples</td>
<td>Negative samples</td>
<td>Positive samples</td>
<td>Negative samples</td>
</tr>
<tr>
<td>Daily</td>
<td>396</td>
<td>1</td>
<td>0</td>
<td>607</td>
</tr>
<tr>
<td></td>
<td>(n=397)</td>
<td></td>
<td></td>
<td>(n=607)</td>
</tr>
<tr>
<td>Weekly</td>
<td>305</td>
<td>22</td>
<td>6</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>(n=327)</td>
<td></td>
<td></td>
<td>(n=252)</td>
</tr>
<tr>
<td>Combined</td>
<td>701</td>
<td>23</td>
<td>6</td>
<td>853</td>
</tr>
<tr>
<td></td>
<td>(n=724)</td>
<td></td>
<td></td>
<td>(n=859)</td>
</tr>
</tbody>
</table>

† A SA IMI was defined as a quarter having at least 1 of the first 3 samples culture positive with $\geq 1$ cfu of SA/0.1mL. Individual quarter samples with $\geq 1$ cfu of SA were considered positive.
CHAPTER 4

VARIATION IN DAILY SHEDDING PATTERN OF \textit{STAPHYLOCOCCUS AUREUS} AND SOMATIC CELL COUNTS IN NATURALLY OCCURRING \textit{STAPHYLOCOCCUS AUREUS} INTRAMAMMARY INFECTIONS

4.1 Introduction

Despite improvements in management, prevention, and treatment, mastitis accounts for 26\% of reported morbidity on US dairies, making it the most prevalent and costly disease of dairy cattle.(Lightner, Miller, Hueston, & Dorn, 1988; USDA, 2007) Dependent on cow traits, stage of lactation, current milk price, cost of replacements, labor and veterinary care, estimates of the cost of mastitis have been calculated at 6\% of the value of production; at 2007 prices that is upwards of 2.1 billion dollars.(Bar et al., 2008; Janzen, 1970; Shim, Shanks, & Morin, 2004) In total, it is estimated that 70-80\% of this loss is due to subclinical intramammary infections (IMI) such as \textit{Staphylococcus aureus} (SA).(Gill, Howard, Leslie, & Lissemore, 1990) The most prevalent contagious mastitis pathogen in the United States, SA was detected in 43\% of bulk tanks examined.(USDA, 2008) Control programs targeting SA have been developed and inconsistently applied throughout the dairy industry.(Fox & Hancock, 1989; Nagahata et al., 2007) Although there have been no published reports explaining the inconsistencies, the cost and perceived inaccuracy of diagnosis are acknowledged hurdles in the broad application of effective control programs.(Studer et al., 2008)
The control of *SA* is contingent on accurate diagnosis of IMI, yet there remains disagreement on what constitutes a definitive diagnosis of a *SA* IMI. Nowhere in mastitis literature is there a definitive standard to diagnose a *SA* IMI. Complicating efforts to define a *SA* IMI is the perception that *SA* is shed intermittently, thus regularly evading diagnosis (Graber et al., 2009; ten Napel et al., 2009). Perhaps the most influential study on the shedding patterns of *SA* was done by Sears et al. who concluded that since *SA* was shed in a cyclical pattern in cows experimentally infected with the Newbould *SA* strain, consecutive samples were necessary for accurate diagnosis. (Sears, Smith, English, Herer, & Gonzalez, 1990) Since then, new technologies such as pulse field gel electrophoresis (PFGE) used to compare strain relatedness, have brought into question the usefulness of *SA* research based on experimental *SA* infections. (Smith et al., 2005) The goal of this study was to describe shedding patterns of naturally occurring *SA* infections over an extended period of time, during three different sampling periods (seasons) throughout a lactation (winter, spring and summer) to provide both a better understanding of the epidemiology of naturally occurring *SA* IMI and to evaluate the impact that shedding patterns may have on detection. In addition, PFGE was used for strain comparisons to evaluate the association of strain type and shedding patterns of bacteria and somatic cell counts (SCC).

### 4.2 Materials and Methods

#### 4.2.1 Study Population and Sampling

Milk samples were collected from 5 multiparous cows (7 quarters) identified with *SA* IMI during the previous lactation and two primiparous cows (two quarters) identified
via quarter level screening of heifers 1 month prior to the study start date. Milk samples were collected for 21 consecutive days, three times throughout the lactation (63 days total, 21 days each during Winter, Spring and Summer). Approximately twelve milliliters of fore-milk was collected from each quarter for culture according to NMC guidelines (NMC, 2004) and a second sample was taken and preserved with bronopol for SCC evaluation at the local Dairy Herd Improvement Association (DHIA) laboratory. Milk samples for culture were kept frozen for up to 22 days and thawed at room temperature for microbiological culture. Disposable, calibrated loops were used to apply 0.01mL of milk on quarter sections of TSA blood agar plates (5% sheep blood, Remel, Lenexa, KS). Plates were incubated at 37˚C and screened at 24 and 48 hours according to NMC (2004) guidelines. A presumptive diagnosis of SA was made based on colony morphology, positive catalase test, presence of hemolysis and a positive coagulase test. Species identification was confirmed at the quarter level by submitting a SA isolate from each SA quarter for identification and antibiotic sensitivity using the TREK Sensititre system. Colony counts (cfu) were recorded from one to 99. All samples with ≥100 cfu/0.01mL were considered too numerous to count. A quarter culture positive with ≥1 cfu/0.01 mL of SA within the first 3 days of the first sample period were designated as quarters with a SA IMI.

4.2.2 Strain Comparisons

A single representative SA isolate from each quarter was submitted for analysis using pulse field gel electrophoresis at The Ohio State University, Infectious Disease Molecular Epidemiology Laboratory (IDMEL). The PFGE protocol used by IDMEL is
adapted from the Canadian protocol created by a subcommittee of The Canadian Committee for the Standardization of Molecular Methods (CCSMM). (Mulvey et al., 2001) Specifically, pure colonies are cultured onto Mueller Hinton agar or Tryptic Soy Agar overnight at 37°C. The following day sterile 5mL culture tubes are labeled according to isolated identification number and filled with 2mL of Staphylococcus Cell Suspension Buffer (CSB) prepared as suggested by Mulvey et al. Cells are then prepared for lysis and 300 μL of the cell solution prepared earlier is placed into the corresponding microcentrifuge tube along with 2 μL of lysostaphin (2μg/mL). A 1.0% agarose gel mixture is prepared using Seakem Gold Agarose (BioWhittaker Molecular Applications, Rockland, ME) and Staphylococcus CSB and is allowed to equilibrate at 50°C before use. Plug gel casts (Bio-Rad Laboratories, Hercules, CA) are labeled to correspond with the isolate. Once the agar has equilibrated, 300 μL of agar are mixed with the cell solution and lysostaphin in the micro-centrifuge tubes. Mixing is achieved by pipetting up and down a few times very quickly. Once mixed, the agar is dispensed into at least two molds using 300μl of the gel solution. Plugs are allowed to solidify for 10 minutes at room temperature. A second set of labeled sterile 1.5ml micro-centrifuge tubes was labeled to correspond to the isolate and filled with 500μl lysis buffer as prepared by Mulvey et al. The solidified gels are removed from the plug molds, placed into the tubes with the lysis buffer, and incubated at 37°C for 1 hr. During this incubation period, proteinase K and PK buffer solution are prepared according to Mulvey et al. Lysis buffer is aspirated from each tube and 500μl of the prepared proteinase K/PK buffer solution is added to each tube. These tubes are incubated at 55°C for at least 30 minutes. Following
this incubation, the plug gels are washed using 0.5X TE buffer prepared according to Mulvey, et al. Plug gels are transferred from the tubes to individual screw caps and washed for at least 90 minutes at 37°C. The Pumpdrive 5206 (Heidolph, Schwabach, Germany) is used to circulate the washing buffer continuously through the sieved screw caps. Plug gels are stored in 1.5ml of the 0.5X TE buffer and refrigerated at 4°C until needed. Restriction digestion is performed using SmaI restriction enzyme. Restriction enzyme buffer (REB) is prepared according to Mulvey et al., and 100μL of this mixture is dispensed into each tube. Two pieces measuring approximately 2mm from the end of the plug gel for each isolate are placed into the REB mixture in the corresponding micro-centrifuge tube. These tubes are incubated at 25°C for 10 minutes. Following incubation, the mixture is aspirated off the gel plugs, and 100μL of REB with SmaI added is dispensed into each tube. Incubation is at 25°C for at least 2 hours. Restriction digestion was performed using XbaI as the restriction enzyme. Incubation temperature for XbaI is 37°C. Following digestion, the plug gel pieces are placed onto the edge of the gel casting comb and allowed to set for 10-15 minutes. Seakem Gold Agarose gel is used to prepare 100mL of 1% agarose gel, which is equilibrated at 50°C. Once equilibrated, the agarose is poured into the gel cast around the gel plug pieces and is allowed to set for 30-45 minutes. The IDMEL uses a CHEF DR III PFGE system (Bio-Rad Laboratories, Hercules, CA). Prior to placing the gel in the chamber, the machine is filled with 2.2L of 1X TBE buffer and allowed to cool to 14°C. Once the gel is solidified and the buffer is cooled to 14°C, the gel is placed into the chamber. The gel is run for 19 hours with an initial switch time of 5.3s, final switch time of 34.9s, included angle of 120, and voltage
of 6V/cm. The gel was developed in 300mL of distilled water with 30μL of ethidium bromide (10mg/mL) for at least 30 minutes. De-staining was performed using 300mL of distilled water for at least 15 minutes. Bands were visualized using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA) and the Quantity One software (Bio-Rad Laboratories, Hercules, CA). The relatedness of the fingerprints was evaluated using Bionumerics software version 4.6 (Applied Maths, Austin Texas) and visual inspection. Position tolerances were set at 1.5% and isolates were grouped into PFGE clusters using Dice coefficients and ≥80% relatedness according to Tenover et al. (Tenover et al., 2008) Related clusters were identified using a numerical designation (1-2), with uppercase letters representing sub-clusters of patterns with ≥90% similarity.

4.2.3 Statistical Analysis

A SA IMI was defined as a quarter positive with ≥1cfu of SA/0.01ml once within the first three days of the first 21-day sample period (winter). Initial exploratory analysis including summary statistics, scatter plots of cfu shed per day per quarter was done to aid in developing a modeling approach (STATA Corp, College Station, Texas). Adapting the categorization of colony count used by Sears et al. (1990), quarters were categorized as low or high shedding quarters according to the mean cfu for the first 21 days. Quarters with a mean 21day SA cfu ≤20 cfu/0.01mL were categorized as “low shedders” and quarters with a mean SA cfu >20 cfu/0.01mL were categorized as “high shedders”. As the cfu counts were not distributed normally, quarters were also classified similarly as high and low shedders according to their median cfu/0.01mL. To account for the non-
Gaussian distribution of cfu counts, a log normal transformation was applied for the evaluation of longitudinal shedding patterns and in evaluating the association of PFGE pulsotype with bacterial shedding, and daily changes in shedding. Samples culturing negative, with a cfu of zero, were randomly assigned a value between 0.1 and 0.5 to allow for the log transformation. Somatic cell counts were converted to linear scores (LS = [(LN(SCC/100)/0.693147)+3]) and categorized into a low (LS≤ 4.00, equivalent to 200,000 SCC/mL) and high (LS> 4.00) SCC group, based on the average LS of the first 21 days. The percentage change from one sample to the next was calculated for ln(cfu) and LS for each sample to examine the extent of the correlation between daily changes in SA cfu shedding and SCC (correlate, STATA v.10). Longitudinal shedding patterns of SA over time were examined for each sample period separately (21 days) and entirely (63 days) according to Studer et al. applying a bandwidth window equivalent to a 6.5 day and a 21 day sample period (lowess, STATA v.10).(Studer et al., 2008) Wilcoxon Rank Sum test (ranksum, STATA) was used to compare ln(cfu)/0.01mL and LS of the designated PFGE pulsotypes.

4.3 Results

A total of 397 milk samples from SA infected quarters were collected for microbiological examination, 388 (97.7%) testing positive (≥1cfu/0.01ml) for SA. Due to culling, only four of the original seven SA infected cows remained through the entire study, and were sampled a total of 63 days, thereby limiting our ability to make comparisons across the three sample seasons. Of the cows that were culled, one was sampled for 42 days and two were sampled for 21 days. The average, median and
standard deviations for days in milk (dim), milk, cfu, ln(cfu) and LS for the group as well as by parity and PFGE pulsotypes are presented in table 4.1. Whether categorized according to average or median cfu over the first 21 days, all quarters were classified as “high” shedders (i.e. shedding a mean/median cfu ≥20cfu/0.01mL). Collectively, over the 3 seasons, 31 (7.8%) of the samples had ≤10 cfu/0.01mL, while 24 (6%) had between 11-19 cfu/0.01ml, leaving 342 (86%) samples with greater than ≥20 cfu/0.01mL. In total, 183 (46%) of the samples had ≥100 cfu/0.01mL. The median LS was 7.8 with a minimum of 0.4 and a maximum of 9.6.

4.3.1 Bacterial Shedding Patterns

As illustrated in figure 4.1, although the amount of shedding varied greatly, 97.7% of the samples were culture positive. Therefore, the ability to detect SA from day to day was very consistent. Using the lowess smoother (lowess, STATA v.10) to carry out a locally weighted regression to evaluate shedding patterns over 6.5 day intervals, there were notable differences in SA shedding patterns between cows as well as within cows; however, no consistent cyclic pattern was identified (Figure 4.2). While quarters 957-RF and 970-RR each appeared to have a sinusoidal shedding pattern, the duration of each cycle differed. Examining quarters 884-RF and 29-RF, no clear pattern was identified, while remaining quarters had a relatively stable shedding pattern. Examining quarters of the 4 cows that were sampled during all 3 sample periods (63 days total), there was no consistent trend over either a 6.5 day period or 21-day period (Figure 4.3). While a repeated sinusoidal trend is noted in 853-G, the amplitude as well as duration of the pattern varies, and shedding was always at a microbiologically detectable limit
(≥1cfu/0.01mL). Figures 4.4 and 4.5 illustrate the shedding patterns of cows 884 & 970, each with 2 SA infected quarters. The remarkable difference in shedding patterns within cows is clear, with each quarter shedding consistently detectable levels of bacteria while exhibiting 4 distinct shedding patterns.

4.3.2 Somatic Cell Count

In total, there were only 10 samples from three SA infected quarters with an LS ≤4 (SCC ≤200,000 cells/ml), all occurring less than 100 days in milk. Using the average linear score for the first 21 days, all quarters were classified as high SCC quarters (LS>4.0). Line graphs were generated for each quarter for the first 21 days of sampling (Figure 4.6) and for the four SA positive quarters sampled for the 3 sample periods (Figure 4.7) to allow for visual examination of trends in daily LS and possible correlation between changes in bacterial counts and SCC. Although it appeared graphically that there might be some correlation between changes in the amount of bacteria shed and the LS, there was little actual correlation. The correlation between the daily change in ln(cfu) and LS for each quarter is presented in table 4.2. The average correlation between the percent change in ln(cfu) and LS was 0.003 (range -0.29 to 0.38). There was also consideration that there might be a “lag” or rebound in either cfu or LS. Examining that possibility, correlations were calculated for ln(cfu) values one and two days post LS in addition to LS values 1 day after ln(cfu). There was no apparent correlation between ln(cfu) and LS using this comparison and there appeared to be no consistent relationship between ln(cfu) and LS.
4.3.3 Strain Differences

The nine isolates were grouped at greater than 80% similarity into 2 clusters, type 1 being further divided into subtype 1-A and 1-B at >90% similarity (figure 4.8). As there was a single isolate representing group 1-B, evaluation of the effects of PFGE pulsotype on shedding of SA and SCC were evaluated at the primary cluster level only (pulsotype 1 and pulsotype 2). The ATCC Newbould strain was also subjected to PFGE and was grouped with the pulsotype 1 isolates at 80% similarity, and into a separate subtype (1-C) when evaluated at >90% similarity. Isolates in the pulsotype 1 group appeared to shed at consistently higher levels with a median cfu/0.01mL of 100 (ln cfu=4.6) at approximately the same level (no trend) throughout all sample periods. Pulsotype 2 isolates had a median of 59 cfu/0.01ml (ln cfu=4.0) and varied shedding patterns over each sample period (Figure 4.9). Comparing ln(cfu/0.01mL) between PFGE pulsotypes, the two-sample Wilcoxon rank-sum test revealed a probability of 0.64 that the ln(cfu) for quarters infected with pulsotype 1 was greater than quarters infected with pulsotype 2 (p=0.000). Similarly, the probability that the LS of quarters infected with isolates in PFGE pulsotype 1 was greater than quarters infected with isolates in pulsotype 2 was 0.59 (p=0.004).

Examining shedding patterns by each sample season we found that, overall, quarters infected with SA clustered into pulsotype 1 shed at more consistent levels than those clustered in pulsotype 2, although pulsotype 2 revealed no consistent pattern over the 3 separate seasons (Fall, Winter spring)(Figure 4.9).
4.4 Discussion

Reviewing SA specifically, there have been three published studies examining the behavior of SA over varied periods of time. In one of the most comprehensive papers published on the diagnosis of mastitis by bacteriological methods, Neave examined 206 SA infected quarters up to 20 times. (Neave, 1973) Unfortunately, the report offered little in regards to explaining the frequency of sampling, making the time period between sample collections unclear, thus allowing limited application of the published findings. The most influential study on the diagnosis of SA to date was done over 28 days by Sears et al. (1990) who examined 19 quarters in 7 cows with experimental SA infections in addition to four naturally occurring SA infections over 16 days. In their report Sears et al. described an apparent cyclical shedding pattern of SA in 16 low shedding quarters (mean cfu ≤10cfu/0.01mL) experimentally infected with the ATCC isolate 29740 (Newbould Strain). From that it was concluded that SA was shed in a cyclical manner and therefore, consecutive samples are necessary for an accurate diagnosis of SA IMI. Since its original report, the “cyclic” shedding behavior of SA has often been described as “intermittent” leaving many with the impression that quarters infected with SA frequently do not shed bacteria at any level. (Graber, Casey, Naskova, Steiner, & Schaeren, 2007; ten Napel et al., 2009) This is understandable as the experimental infections examined by Sears et al. were culture-positive only 75% of the time. However, it is important to consider that while 84% of the experimental quarters examined by Sears et al. were classified as “low shedders”, only 50% of the natural infections (n=4) were classified similarly and were culture positive 89% of the time. Following Sears’ study Daley et al., again using the Newbould strain, in 10 cows over 5 days, reported similar findings in addition to the...
observation that the SCC of infected quarters exhibited an inverse cycling relationship to the shedding of bacteria. (Daley, Oldham, Williams, & Coyle, 1991) Daley et al. (1991) also noted that the frequency, periodicity and amplitude of the cycle over the five-day period were independent for each infected quarter. Although these studies employing experimental infections of the Newbould strain have been the foundation of SA IMI diagnostics, recent studies using multilocus sequence typing have since revealed that the Newbould strain of SA used in these experimental infections appears to have low affinity for the udder and are more similar to skin isolates than those recovered from infected mammary glands. (Smith et al., 2005) While it is difficult then to accept the conclusions drawn from Sears’ study examining experimental infections caused by the Newbould-SA strain, those drawn from the four natural infections (sampled for 16 days) remain of interest. Of the 4 natural infections studied by Sears, two were classified as “high shedders” (mean cfu ≥20 cfu/0.01mL), shedding detectable levels 100% of the time. The two quarters categorized as “low shedders” shed detectable levels 93% and 63% of the time, respectively. It is important to note that, unlike the experimental infections which were cultured using a 0.1 mL inoculum and considered positive at ≥0.1cfu/1mL, cows with natural infections were examined using a 0.05mL inoculum and were considered positive with >1 cfu/0.05mL. As the difference in microbiologic protocol may have had significant impact on the sensitivity of detection, it is difficult to compare the findings from the natural and experimental infections. In comparison to the study by Sears et al., none of the quarters examined in our study qualified as “low shedders”, the lowest mean cfu being 42 cfu/0.01 mL, 7 cfu above what Sears considered too numerous to count. Recently, Studer et al. (2008) examined 11 natural SA IMI over 14 days and reported
91.6% (using a 0.1mL inoculum) of the samples were culture positive (at ≥ 3cfu/0.1mL) and that 6 of the 11 quarters exhibited a sinusoidal shedding pattern, the length of which was 6.5 days. Two additional patterns, log-linear increasing and no trend, were also noted. Although we were able to document a sinusoidal type pattern in some quarters over a single 21-day period and over the 3 sample seasons combined, the amplitudes and durations varied such that a true “cycle” could not be defined between or within cows. Just as Daley et al. (1991) and Studer at al. (2008) found a within cow difference in shedding patterns, a similar independence was noted within cows in our study cows 884 and 970, each having two SA infected quarters and revealing 4 different shedding patterns (Figures 4.4 & 4.5). As only 2 of the 9 quarters were grouped into PFGE pulsotype 2, conclusions drawn should be made with caution. Of interest is that the ATCC Newbould strain was found to be 80% similar to the isolates in PFGE pulsotype 1. While infections “typical” of the Newbould strain were reported to have only moderate increases in SCC (Studer et al., 2008), with 16 of 19 quarters in the Sears (1990) study shedding ≤10cfu/0.01ml; quarters in our study infected with SA clustered into PFGE pulsotype 1, shed at consistently high levels with a median cfu/0.01mL of 100 and a median LS of 7.8 compared to type 2 with a median cfu/0.01 mL of 59 and an LS of 7.4.

In evaluating the extreme differences in individual shedding patterns across cows, within cows and even within quarters across sample periods, an important consideration should be made in reflecting on the effect of sample collection and processing. As it is virtually impossible to collect a sterile sample truly representative of the entire milking, it is equally impossible to examine the total volume of milk microbiologically. The fact that 0.01 to 0.1mL of milk was used to represent the total pounds of milk produced at
single milking may have a significant impact on the perceived “cyclical” variation in bacterial shedding. Therefore, while general trends (high or low median cfu or LS) may be explained by strain relatedness, actual variation may be best explained by sample collection and processing and not by within cow and between cow differences in infection duration, strain type or immune function. Although this sample variation is inevitable, it highlights the need for consistency for the remainder of all related procedures, from sample collection, sample volume, inoculum volume, and plate counting so that future comparisons are at least contextually possible. Such consistency does not currently exist and due to the perceived intermittent shedding of SA, there have been variable prerequisites applied to screening for and the confirmation of SA IMI. NMC’s published guidelines suggest that 2 of 3 consecutive cultures should be positive for a quarter to be considered infected. (NMC, 1999) Although such requirements may be well suited to research conducted in experimental settings, they are cumbersome and cost prohibitive in field studies and day-to-day mastitis diagnostics. In our study, the ability to detect SA using microbiological culture was tremendously consistent. Although shedding varied tremendously in both number of cfu and pattern, it appears that such patterns are of little consequence in the accurate detection of SA IMI in these quarters. Erskine reported that percentage agreement between duplicate samples was highest for contagious pathogens such as SA (94.2%), suggesting that a single quarter sample might be adequate in determining infection status. (Erskine & Eberhart, 1988) Our study examining naturally occurring infections would support Erskine’s conclusion.

Several limitations are noted in the data that was collected. As several cows were culled prior to the completion of the study, there was limited data available to reliably
examine the effects of season or days in milk on shedding patterns of SA or LS. Microbiological culture limited the maximum colony count to 100 CFU’s per 0.01ml sample. In total, 46% of the samples exhibited growth exceeding 100 CFU/0.01ml. Although such values certainly do not impede diagnosis, our ability to track the true extent of variability in the shedding at higher levels was limited as a great deal of that information was essentially lost. Although far less influential (n=25), a similar effect was seen in the reporting of SCC’s. The machine’s reportable limit is 10 million cells/ml, therefore a similar ceiling effect was found in the SCC data, which limited the evaluation of daily changes in LS. While Studer et al. identified three shedding patterns amongst 11 quarters using cell equivalents obtained using real-time quantitative PCR, we were unable to identify any predictable shedding pattern of SA using microbiological culture results. As this study was done on a single dairy, results may not be representative of other strains of SA such as the “low shedding” natural infections noted by Sears et al. (1990), although as noted (Figure 4.8) the Newbould strain was grouped in to one of our strain pulsotypes at >80% similarity (an individual sub-type at ≥90% similarity). Nevertheless, much can be gained from this examination of naturally occurring SA infections over such an extended sample period. As far back as 1973, a call was made for science to reach an agreement as to what actually defines “mastitis” in terms of cell count or culture.(Neave, 1973) Neave pointed out then that the definitions of mastitis were “not suitable” and “cumbersome” and wrote that the then current definition of “normal” was too rigid. As such an agreement has yet to be accomplished, little if any continuity or comparability between published studies is possible. Although this single study will surely not answer all of our questions regarding diagnostic criterion for all IMI, it is one of few that have
examined naturally occurring SA IMI for an extended period of time. Future studies examining naturally infected quarters over longer periods, in addition to examining the genetic relatedness of strains and shedding patterns, may further aid in developing more coherent prerequisites for the diagnosis of SA IMI.
4.5 References

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Table 4.1. Days in milk (dim), milk production in pounds (milk), cfu/0.01mL, ln(cfu) and linear score (LS) for all quarters, by parity (Lactation) and by PFGE pulsortype.

<table>
<thead>
<tr>
<th></th>
<th>DIM</th>
<th>MILK</th>
<th>cfu/0.01mL</th>
<th>ln(cfu)</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Qtrs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>163</td>
<td>74.8</td>
<td>69.4</td>
<td>3.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Median</td>
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<td>78.9</td>
<td>90</td>
<td>4.5</td>
<td>7.7</td>
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<td>19.8</td>
<td>35.3</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>By Parity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactation 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
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<td>68.1</td>
<td>75.2</td>
<td>4.0</td>
<td>7.3</td>
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<tr>
<td>Median</td>
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<td>98</td>
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<td>7.5</td>
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<tr>
<td>SD</td>
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<td>33.0</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Lactation 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>124</td>
<td>86.4</td>
<td>65.7</td>
<td>3.9</td>
<td>8.0</td>
</tr>
<tr>
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<td>72</td>
<td>4.3</td>
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<td>SD</td>
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<td>0.81</td>
<td>1.1</td>
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<tr>
<td>Lactation 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>168</td>
<td>71.7</td>
<td>674</td>
<td>3.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Median</td>
<td>138</td>
<td>77.3</td>
<td>97</td>
<td>4.6</td>
<td>7.5</td>
</tr>
<tr>
<td>SD</td>
<td>80</td>
<td>27.3</td>
<td>36.9</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>By PFGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFGE 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>144</td>
<td>80.6</td>
<td>74.9</td>
<td>4.1</td>
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<tr>
<td>Median</td>
<td>120</td>
<td>81.9</td>
<td>100</td>
<td>4.6</td>
<td>7.8</td>
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<tr>
<td>SD</td>
<td>59.3</td>
<td>11.7</td>
<td>33.3</td>
<td>0.99</td>
<td>1.5</td>
</tr>
<tr>
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<td>64.1</td>
<td>57.2</td>
<td>3.6</td>
<td>7.3</td>
</tr>
<tr>
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<td>69.7</td>
<td>59.0</td>
<td>4.1</td>
<td>7.4</td>
</tr>
<tr>
<td>SD</td>
<td>87</td>
<td>26.2</td>
<td>36.4</td>
<td>1.4</td>
<td>1.4</td>
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Figure 4.1. Daily bacterial counts of SA (cfu/0.01mL) over the entire study period (63 days). Each individual graph designated by Cow ID number and quarter (RF=Right Front, RR=Right Rear, LF=Left Front, LR=Left Rear).
Figure 4.2. Lowess smoother of ln(cfu/0.01mL) for each quarter over a 6.5 day window for Season 1-Winter (the first 21 day sample period). Individual graphs designated by Cow ID number and quarter (RF=Right Front, RR=Right Rear, LF=Left Front, LR=Left Rear).
Fig 4.3. Lowess smoothing curves of ln(cfu/0.01mL) over three separate 21 sample periods evaluated over a 6.5 day window (bandwidth=0.1, LEFT) and a 21 day window (bandwidth=0.33, RIGHT) for the 4 quarters sampled over 3 separate seasons, Winter-Spring-Summer (63 days total)
Figures 4.4. Lowess smoother for ln(cfu)/0.01mL over a 6.5 day window for the first 21 day sample period, Season 1 (Winter), in 2 quarters of cow 884-L.(RF= Right Front quarter, RR= Right Rear Quarter)
Figures 4.5. Lowess smoother for ln(cfu)/0.01mL over a 6.5 day window for the first 21 day sample period, Season 1 (Winter), in 2 quarters of cow 970-T. (LR= Left Rear quarter, RR= Right Rear Quarter)
Figure 4.6: Daily cfu/0.01mL and Linear Score (LS) for each SA infected quarter over the first 21 sample period. Each graph designated by Cows ID and quarter (RF=Right Front, RR=Right Rear, LF=Left Front, LR=Left Rear).
Figure 4.7. Daily cfu/0.01 mL and linear score (LS) for quarters sampled over 3 seasons (Winter-Spring-Summer). Each graph represent an individual quarter labeled with cow id and quarter.
Table 4.2. Correlation between percentage daily change in ln(cfu) and linear score (LS)

<table>
<thead>
<tr>
<th>Quarter</th>
<th>PFGE type</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>29-D</td>
<td>2</td>
<td>-0.29</td>
</tr>
<tr>
<td>32-E</td>
<td>1</td>
<td>-0.23</td>
</tr>
<tr>
<td>853-G</td>
<td>1</td>
<td>0.19</td>
</tr>
<tr>
<td>957-Q</td>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
<td>966-S</td>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>884-L RF</td>
<td>1</td>
<td>-0.13</td>
</tr>
<tr>
<td>884-L RR</td>
<td>1</td>
<td>0.006</td>
</tr>
<tr>
<td>970-T RR</td>
<td>1</td>
<td>0.38</td>
</tr>
<tr>
<td>970-T LR</td>
<td>1</td>
<td>0.34</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.003</td>
</tr>
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Figure. 4.8 PFGE of representative isolates from each SA infected quarter. Position Tolerances were set at 1.5% and isolates were grouped into PFGE clusters using Dice coefficients and ≥80% relatedness. Related clusters were identified using a numerical designation (1-2), with uppercase letters representing sub-clusters of patterns with ≥90% similarity.
Figure 4.9. Daily ln(cfu)/0.01 ml by PFGE pulsotype for each 21 sample period (Season 1, 2 and 3). Each panel has a graph designated by PFGE pulsotype (1 and 2).
CHAPTER 5

SHEDDING PATTERNS OF NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS
INTRAMAMMARY INFECTIONS SAMPLED WEEKLY THROUGHOUT LACTATION

5.1 Introduction

Despite improvements in management, prevention, and treatment, mastitis accounts for 26% of reported morbidity on US dairies making it the most prevalent and costly disease of dairy cattle. (Lightner, Miller, Hueston, & Dorn, 1988; USDA, 2007) Dependent on cow traits, stage of lactation, current milk price, cost of replacements, labor and veterinary care, estimates of the cost of mastitis have been calculated at 6% of the value of milk production; at 2007 prices that is upwards of 2.1 billion dollars.(Bar et al., 2008; Janzen, 1970; Shim, Shanks, & Morin, 2004) In total, it is estimated that 70-80% of this loss is due to subclinical intramammary infections (IMI) such as Staphylococcus aureus (SA).(Gill, Howard, Leslie, & Lissemore, 1990) The most prevalent contagious mastitis pathogen in the United States, SA was detected in 43% of bulk tanks examined.(USDA, 2008) Control programs targeting SA have been developed and inconsistently applied throughout the dairy industry.(Fox & Hancock, 1989; Nagahata et al., 2007) Although there have been no published reports explaining the reason for the inconsistencies, the cost and perceived inaccuracy of diagnosis are
acknowledged hurdles in the broad application of effective control programs. (Studer et al., 2008)

The control of SA is contingent on accurate diagnosis of IMI, yet there remains disagreement on what constitutes a definitive diagnosis of a SA IMI. Nowhere in mastitis literature is there a definitive standard to diagnose SA IMI, although recent recommendations have been published. (Torres, Rajala-Schultz, & DeGraves, 2009) Sears et al. concluded that since SA was shed in a cyclical pattern in cows experimentally infected with the Newbould SA strain, consecutive samples were necessary to accurately diagnosis a SA IMI. (Sears, Smith, English, Herer, & Gonzalez, 1990) Since then, new technologies, such as pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) used to compare strain relatedness, have brought into question the usefulness of SA research based on experimental SA infections. (Smith et al., 2005) The goal of this study was to evaluate bacterial shedding patterns of SA, specifically the influence of clonal relatedness of SA on the shedding patterns of bacteria and SCC in naturally occurring SA infections, from the beginning to the end of lactation, to provide a better understanding of the epidemiology of naturally occurring SA IMI and to evaluate the impact shedding patterns might have on detection.

5.2 Materials and Methods

5.2.1 Study Population and Sampling

Quarter milk samples were collected weekly from lactating cows at 2 dairies. Based on previous composite level herd culture results and follow up quarter level screening, 16 cows (22 quarters) were confirmed to have at least 1 quarter culture
positive for SA (≥1cfu/0.01mL) on 2 separate sample weeks prior to dry off and were included for sample collection during the proceeding lactation. Every quarter of every cow was treated with an approved dry cow tube (Orbenin-DC or Quartermaster) at the end of lactation. An additional 16 first lactation heifers (24 quarters) with milk samples that were culture positive (≥1cfu/0.01mL) for SA within the first 7 days of calving were also enrolled in the study. A SA IMI was defined as a quarter having at least 1 out of the first four consecutive samples culture positive with ≥1cfu of SA/0.01mL. Individual quarter samples were considered positive when ≥1 cfu/0.01mL was recovered microbiologically. (Torres, Rajala-Schultz, & DeGraves, 2009) Individual quarters samples were collected from every functional quarter of every cow once a week beginning the first week of lactation for up to 44 weeks. Daily milk weights in addition to monthly composite test-day information were also recorded.

Approximately 12 mL of foremilk was collected from each quarter for culture, according to NMC guidelines, and kept frozen until cultured. (NMC, 2004) A second sample was taken, kept refrigerated and preserved with bronopol and submitted for SCC evaluation (Fossomatic cell counter, Scanco, Denmark) at the local Dairy Herd Improvement Association laboratory (DHIA) within 84 hours of sample collection. Quarter milk samples for SCC evaluation were not collected from first lactation heifers in the first sample week on the second dairy due to parlor time constraints, but were collected weekly thereafter. Milk samples for culture were kept frozen for up to 10 days and thawed at room temperature for microbiological culture. To allow for accurate colony counts, 0.01mL of milk was applied to individual TSA blood agar plates (5% sheep blood, Remel, Lenexa, KS) using disposable, calibrated loops (Nunc, Fisher
Plates were incubated at 37˚C and screened at 24 and 48 hours according to NMC (2004) guidelines. A presumptive diagnosis of SA was made based on colony morphology, positive catalase test, presence of hemolysis and a positive coagulase test. Colony morphology, color and hemolysis were recorded. SA isolates exhibiting different hemolytic patterns, color or size within the same sample were isolated separately and recorded as isolates “a” and “b”. Three isolates from each quarter, representing cultures from early, mid and late lactation, in addition to a representative isolate from the preceding lactation collected prior to dry-off were confirmed as SA species using the Trek automated Sensititre identification system. (Trek diagnostic Systems, Cleveland, OH) Antimicrobial minimum inhibitory concentrations (MIC) were also evaluated using the automated Trek Sensititre mastitis plate (CMV1AAMAF) for each isolate submitted. When a SA quarter was culture negative for more than 3 weeks in a row and SA was again recovered, then additional isolates were submitted for PFGE and sensitivity. When SA colony morphology was distinctly different within the same sample, each “a” and “b” isolate was submitted separately for identification and MIC. Milk samples that were culture negative for SA or culture positive with a single colony of SA using a 0.01 mL inoculum from quarters that had been confirmed SA positive were thawed a second time and cultured using a larger volume of milk (0.1mL).

5.2.2 Strain Comparisons

Isolates identical to those submitted for ID and sensitivity, including a representative SA isolate collected in the preceding lactation prior to dry off, were
submitted for analysis using pulse field gel electrophoresis at The Ohio State University, Infectious Disease Molecular Epidemiology Laboratory (IDMEL). The PFGE protocol used by IDMEL is adapted from the Canadian protocol created by a subcommittee of The Canadian Committee for the Standardization of Molecular Methods (CCSMM) as described in Chapter 4. If a SA quarter was culture negative for more than 3 weeks, the SA isolate from the next SA positive culture was submitted for PFGE. When SA colony morphology was distinctly different within the same sample, each “a” and “b” isolate were submitted separately for PFGE. In addition, the ATCC Newbould strain was submitted for comparison. The PFGE protocol used by IDMEL is adapted from the Canadian protocol created by a subcommittee of The Canadian Committee for the Standardization of Molecular Methods (CCSMM). (Mulvey et al., 2001) A dendrogram of SA isolates was developed using Bionumerics software version 4.6 (Applied Maths, Austin, TX). Position tolerances were set at 1.5% and isolates were grouped into PFGE clusters based on Dice coefficients and >80% relatedness according to Tenover et al. (Tenover et al., 2008). Related clusters were identified using numerical designations (1-3), with upper case letters (A-C) representing sub-clusters of patterns with ≥ 90% similarity. Isolates from individual quarters were inspected and compared at the quarter level to assess the similarity of PFGE patterns of isolates from individual quarters throughout lactation.

5.2.3 Statistical analysis

A sample was considered culture positive for SA with ≥1 cfu/0.01mL. Analysis was performed at the quarter level: right front (RF), right rear (RR), left front (LF) and
left rear (LR). Each quarter sample was considered independently. Quarters included in the analysis were those that were sampled for at least 26 weeks and culture positive for SA at least once within the first 4 weeks of sampling. Additional quarters excluded from the analysis included those which tested positive for SA in the previous lactation, but failed to test positive within the first 4 weeks (cures) of the lactation and quarters in cows that were treated with intramammary or systemic antibiotics at anytime during the current lactation. Quarters with no history of SA, but testing positive for SA after the fourth week, were considered new infections and not included in the analysis (new, n=7). Milk samples that were unreadable by the Fossomatic counter due to coagulation (n=8) were recorded with the maximum value (9999). Exploratory analysis, including summary statistics, scatter plots of scc and cfu cultured per sample (week) per quarter was done to examine the distribution of cfu and SCC values. (STATA Corp, College Station, Texas) Adapting the categorization of colony count used by Sears et al. (1990), quarters were categorized as high or low shedding quarters according to the mean cfu/0.01mL of each quarter. Quarters with a mean SA cfu ≤ 20 cfu/0.01mL were categorized as “low shedders” and quarters with a mean cfu ≥20 cfu/0.01mL were categorized as “high shedders”. As the cfu counts were not distributed normally, quarters were also classified similarly as high and low shedders according to their median cfu/0.01mL. To account for the non-Gaussian distribution of cfu counts, a log normal transformation was applied for the evaluation of longitudinal shedding patterns. Culture negative samples with zero cfu were randomly assigned a value between 0.1 and 0.5, to allow for the log transformation. Somatic cell count (SCC) for weekly samples, as well as monthly composite test-day information, were converted to linear scores (LS) using the formula (LS =
\[
[(\ln(\text{SCC}/100)/0.693147)+3]\]
and categorized into low (LS \(\leq 4.0\), equivalent to 200,000 SCC/mL) and high SCC (LS >4.0) groups based on the average LS of the lactation. Longitudinal shedding patterns of \(S.\alpha\) over an 8-week window (bandwidth=0.18) were examined using the lowess smoother application (lowess, STATA Corp, College Station, Texas). Wilcoxon Rank Sum test (ranksum, STATA) was used to compare the \(\ln(\text{cfu})/0.01\text{mL}\) and the LS of designated PFGE types. Comparisons with p-values \(\leq 0.05\) were considered to be significantly different. In addition, as there was a single cow represented in PFGE group 3 it was excluded from the regression analysis.

5.3 Results

Of the 16 cows identified in the preceding lactation with \(S.\alpha\) IMI, 8 cows (9 quarters) were sampled a minimum of 26 weeks and included in the analysis. Of the 29 quarters included in the analysis, 6 were from second lactation cows and 3 were from third or greater lactation cows. A total of 14 heifers were included in the analysis (20 quarters), bringing the total number of quarters included in the analysis to 29. In addition to quarters not sampled for at least 26 weeks (n=5), other quarters excluded from the analysis included those which tested positive for \(S.\alpha\) in the previous lactation, but failed to test positive within the first 4 weeks (cure, n=8) and quarters culture positive for \(S.\alpha\) in cows that were treated with intramammary or systemic antibiotics at anytime during the current lactation (treated, n=2). Of the 29 quarters included in the analysis, 20 were sampled \(\geq 36\) weeks with an average sample coverage of 37 weeks. In all, 1070 milk samples were collected for microbiological examination, while 1049 milk samples were evaluated for SCC. A summary of quarter level values of cfu, scc, test-day scc and days
positive with 0.01 and 0.1mL inoculums are presented in table 5.1. Using the 0.01mL inoculum, 914 of the 1070 samples (85%) were culture positive at ≥1cfu/0.01mL. Using the larger 0.1mL inoculum, 1011 of the 1070 (95%) samples were culture positive at ≥1cfu/0.1mL. Of the 59 samples that were culture negative at the larger inoculum volume, 40 (68%) were represented by 3 quarters in 2 heifers. The average, median and standard deviations for milk, cfu, ln(cfu), LS and test-day LS are presented in table 5.2 by the entire cohort, by parity, and by PFGE type. When categorized by mean cfu/0.01mL, there were no quarters classified as “low shedders” (mean cfu ≤20cfu/0.01mL). In total, 344 (32%) of all samples had ≤ 10cfu/mL. However, using the median cfu, there were 12 quarters with a median cfu ≤ 20cfu/0.01mL. The median LS was 6.4 with a minimum of 0 and a maximum of 9.6. The median test-day SCC was 460,000 (LS=5.2). Examining hemolysis, 55% of the isolates exhibited only incomplete hemolysis, 29% exhibited both complete and incomplete hemolysis, 8% exhibited only complete hemolysis, 7% exhibited 2 different types (complete with incomplete and only incomplete) of hemolysis and 1% had hemolysis limited to directly beneath the colony.

5.3.1 Bacterial Shedding Patterns

As shown in figure 5.1, evaluating shedding patterns using the lowess smoother to carry out a locally weighted regression, there are notable differences in SA shedding patterns between cows as well as within cows. While a sinusoidal type pattern may appear to be present in many quarters (4401-LF, 1574-LF, 1384 LR/RR and 115-RR), the duration and amplitude of each varies such that no consistent pattern can be identified between or within cows. This is best seen comparing both quarters of cow 1384, each
having a sinusoidal type pattern, each with 5 peaks but at varied points in time, the LR at weeks 3, 16, 22, 26 and 32 and the RR at weeks 3, 14, 22, 34 and 40 (figure 5.2). In contrast, other quarters seem to have a stable shedding pattern with comparatively little to no variability (Figure 5.1, quarters: 115-LR, 74-LF, 4564-LF, 4320-RF). Of particular interest are the differences in shedding patterns within cows with 2 or more infected quarters. Extreme within-cow variation is clear when examining weekly shedding patterns for cows 101 (Figure 5.3) and 115 (Figure 5.4) each with 2 or more infected quarters. Cow 101 (Figure 5.3) had two infected quarters, the LF shedding at a consistently higher level (median cfu=317) and culturing positive for SA 43/44 (98%) of the time while the RF quarter shed less consistently with a median cfu of 1/0.01mL and tested culture positive 64% of the time (84% when using the 0.1mL inoculum). In contrast, cow 115 (Fig 5.4) with three SA positive quarters, while exhibiting three different shedding patterns, tested positive 100% (LR) and 98% (RF and RR) of the time using a 0.01mL inoculum, while all three quarters were culture positive 100% of the time when using the larger, 0.1mL inoculum. As summarized in Table 5.1, despite the variation in both shedding patterns and the amount of shedding from day to day within and between cows, when using the larger inoculum volume (0.1mL), SA was consistently shed at detectable levels with the exception of a few quarters: 1617-LR (77%), 1617-RR (85%) and 4387-LF (39%).

5.3.2 Somatic Cell Counts

The median LS across all quarters was 6.4 (>1,000,000 cells/mL) while the median cow composite test-day SCC was 5.2 (460,000 cells/mL). As shown in table 5.2,
the median quarter LS in heifers was 6.12 and the median quarter LS in second multiparous cows was 7.2. While 28 of the 29 SA quarters were classified as “high” SCC quarters (median LS ≥4), a single quarter (4393-LF) was classified as a “low” SCC quarter with a median LS of 3.2. Of all the samples from SA positive quarters evaluated for SCC, 128 (12%) had LS ≤4 (200,000 cells/mL). Examining cow level monthly composite test day information that is readily available to dairy personnel for review, 97 of the 187 (52%) monthly cow composite test-day values obtained from SA positive cows in the study had a LS ≤ 5.32 (500,000 cells/mL), a common cut-off used in trying to identify SA infected cows. Examining the median monthly composite test scores across the entire lactation, 17 (59%) of the SA cows had a median composite test-day LS ≤ 5.32 (500,000 cells/mL), 6 of them having a median composite test day ≤ 4 (200,000 cells/mL). Interestingly, at the cow level, the median cow composite test-day LS for multiparous cows (test-day LS=5.3) was similar to primiparous cows (test-day LS=5.2), both under 500,000 cells/mL.

5.3.3 Strain Differences

Examining isolates from individual quarters collected at three separate time points during the lactation, the PFGE banding patterns were consistent, thus supporting the assumption that the SA IMI was persistent and not likely a second new infection. Comparing the PFGE patterns of isolates exhibiting different colony morphology within the same sample (isolates “a” and “b”) revealed pulsotypes that were >95% similar in spite of differences in hemolysis, colony size or color. The isolates collected from the 29 SA positive quarters included in the analysis in addition to the isolates from cured
quarters and the ATCC Newbould strain isolate were grouped into 3 clonal types at >80% similarity, pulsotype 1 being further divided into subtype 1-A, B & C and pulsotype 2 into subtypes 2-A and 2-B at >90% similarity (figure 5.5). Excluding cured quarters, there were a total 16 quarters (11 cows) grouped in PFGE pulsotype 1 and 11 quarters (10 cows) grouped into PFGE pulsotype 2. A single cow, with 2 SA positive quarters was grouped in PFGE pulsotype 3. Grouped by PFGE type and lactation, 11 of the 16 quarters (69%) in PFGE pulsotype 1 were from primiparous cows, while 9 of the 11 quarters (82%) in PFGE pulsotype 2 were from primiparous cows. Of the 5 cows with multiple quarters infected with SA, 4 were grouped in PFGE pulsotype 1. Of the 8 quarters that cured over the dry period, 7 (88%) were in PFGE pulsotype 2. Also shown in figure 5.4, the ATCC Newbould strain was grouped into PFGE pulsotype 1 at >80% similarity, and placed in an individual subtype 1-C when evaluated at 90% similarity.

5.3.3.1. The Effects of Strain Differences on Bacterial Shedding

While isolates from low shedding quarters (median cfu ≤ 20cfu/mL) were evenly split across pulsotypes, quarters clustered in PFGE pulsotype 1 and pulsotype 3, with a median cfu/0.01mL of 83 (ln cfu=4.4) and 348 (ln cfu=5.9) respectively, appeared to shed at consistently higher levels than quarters infected with isolates in the PFGE pulsotype 2 with a median cfu of 17cfu/0.01mL (median ln cfu=2.8) (Table 5.2). As there were only a few quarters representing each sub-pulsotype, evaluation of the effects of PFGE classification on bacterial shedding were evaluated at the primary cluster level only (pulsotypes 1 and 2). Type 3 was excluded as it was represented by only 1 cow (2 quarters). Comparing bacterial shedding between quarters infected with PFGE pulsotypes
1 and 2, the two-sample Wilcoxon Ranked Sum test revealed that, on average, quarters infected with isolated within pulsotype 1 shed higher numbers of bacteria compared to quarters infected with isolates within pulsotype 2 (p < 0.000).

5.3.3.2. The Effects of Strain Differences on SCC

The median LS of quarters shedding isolates grouped into pulsotype 1 was 7.2 (>1,000,000 cells/mL), while the median LS of quarters shedding isolates grouped into pulsotype 2 was 5.0 (400,000 cells/mL). Comparing LS between quarters infected with bacteria in pulsotypes 1 and 2, the two-sample Wilcoxon Ranked Sum test revealed quarters infected with isolates grouped in pulsotype 1 shed higher numbers of cells compared to quarters infected with isolates grouped into pulsotype 2 (p < 0.000). At the cow level, the median monthly composite test-day LS of cows with quarters shedding SA grouped into pulsotype 2 was 4.2 (<250,000 cells/mL) and cows with quarters shedding SA grouped into pulsotypes 1 and 3 had a median monthly composite test-day LS of 6.3 and 7.3 respectively, each over 1,000,000 cells/mL. This is best illustrated in figure 5.7 with a vertical line at a LS of 5.3 (500,000 cells/mL), a common cut-off used by dairy personnel in identifying problem cows for microbiological culture. Figure 5.7 shows that 70% of monthly composite test-day LS values from cows with SA quarters infected with SA grouped into pulsotype 1 tested higher than 5.3 (500,000 cell/mL) and only 20% of the monthly composite test-day LS scores of cows with SA infected quarters shedding SA grouped in pulsotype 2 were greater than 5.3.
5.4 Discussion

Reviewing SA specifically, there have been three published studies examining the behavior of SA, each over relatively short periods of time. In one of the most comprehensive papers published on the diagnosis of mastitis by bacteriological methods, Neave examined 206 SA infected quarters up to 20 times. (Neave, 1973) Unfortunately, the report offered little in regards to explaining the frequency of sampling, making the time period between sample collections unclear, thus allowing limited application of the published findings. To date, the most influential study on the diagnosis of SA was published by Sears et al. (1990). The Sears study examined 19 quarters in 7 cows with experimental SA infections over 28 days, in addition to 4 naturally occurring SA infections over 16 days. In their report, Sears at al. described a cyclical shedding pattern of SA in 16 low shedding quarters (mean cfu ≤ 10cfu/0.01mL) experimentally infected with the ATCC SA isolate 29740 (Newbould Strain). From Sears’ study it was concluded that SA was shed in a cyclical manner and therefore, consecutive samples were necessary for an accurate diagnosis of SA IMI. Following Sears’ study, Daley et al. inoculated 10 cows with the same Newbould strain and examined shedding patterns over 5 days and reported similar findings to Sears, in addition to the observation that SCC of infected quarters exhibited an inverse cycling relationship to the shedding of SA. (Daley, Oldham, Williams, & Coyle, 1991) Daley et al. also noted that the frequency, periodicity and amplitude of the cycle over the five-day period were independent for each infected quarter.

Since the original report by Sears et al. (1990), the “cyclic” shedding behavior of SA has often been described as “intermittent” leaving many with the impression that
quarters infected with SA frequently do not shed bacteria at any level (Graber, Casey, Naskova, Steiner, & Schaeren, 2007; ten Napel et al., 2009). This is understandable as the experimental infections examined by Sears et al. were culture-positive (≥ 1cfu/0.1mL) only 75% of the time. However, it is important to consider that while 84% of the experimental quarters examined by Sears et al. were classified as “low shedders”, only 50% of the natural infections (n=4) were classified similarly and were culture positive 89% of the time. Comparatively, 85% of the samples collected in our study were culture-positive for SA (≥ 1cfu/0.01mL) using a smaller inoculum (0.01mL), while 95% were culture-positive for SA (≥ 1cfu/0.1mL) when using the same inoculum volume (0.1mL) as used by Sears et al. (1990). It is also important to consider that while we are rarely able to establish the duration of natural infections, the majority of experimental infections are of relatively short duration. While some might presume that heifers that freshen with SA IMI have “new” infections when compared to multiparous cows, it is unclear when exactly heifers are becoming infected with SA, and therefore, such assumptions might be faulty, making conclusions drawn from said assumptions dangerous. For that reason, further research is necessary to address the epidemiology of SA IMI in heifer populations. From our work, it is clear that those experimental infections, whether due to the chosen strain or perhaps more likely due to the duration of infections, are not likely to be accurate representations of naturally occurring SA IMI.

Although studies employing experimental infections using the Newbould SA strain have been the foundation of SA IMI diagnostics, recent studies using MLST have since revealed that the Newbould strain of SA used in experimental SA IMI appears to have low affinity for the udder and is more similar to skin isolates than those recovered
from infected mammary glands. (Smith et al., 2005) While this makes it more difficult to accept the conclusions drawn from Sears’ study examining experimental infection caused by the Newbould-SA strain, the observations obtained from the naturally occurring infections (sampled for 16 days) remain of interest. Of the 4 natural infections studied by Sears et al. (1990), 2 were classified as “high shedders” (mean cfu ≥ 20cfu/0.01mL), shedding detectable levels 100% of the time. The two quarters categorized as “low shedders” shed detectable levels 93% and 63% of the time. However, it is important to note that, unlike the experimental infections which were cultured using a 0.1mL inoculum and considered positive at ≥1cfu/0.1mL, cows with natural infections were examined using a 0.05mL inoculum and were considered positive at >1cfu/0.05mL. As the difference in microbiologic protocol might have a significant impact on the sensitivity of detection, it is difficult to compare the findings from the natural and experimental infections. However, it is clear that the naturally infected quarters shed SA in a more consistent pattern than quarters experimentally infected with SA. In comparison to the study by Sears et al. (1990), none of the quarters examined in our study qualified as “low shedders”, the lowest mean cfu being 23 cfu/0.01mL, 13 cfu above what Sears et al. classified as a “low shedder”. Recently Studer et al. (2008) examined 11 natural SA infections over 14 days and reported that 91.6% of the samples (using a 0.1 mL inoculum) were culture positive (≥3 cfu/0.1mL) and that 6 of the 11 quarters exhibited a sinusoidal shedding pattern, the length of which was 6.5 days. Two additional patterns, log-linear increasing and no trend were also noted. As samples in our study were taken every 7 days, we were not able to evaluate the 6.5 day cyclic pattern calculated by Studer et al. Although we were able to document several sinusoidal type patterns in some
quarters over the lactation, as the amplitudes and durations vary, it is difficult to define a true “cycle” between or even within cows. Just as Daley et al. (1991) and Studer et al. (2008) found within-cow differences in shedding patterns, similar independence was noted within cows in our study.

With regard to the effects of strain type, it is of interest that of the quarters included in the analysis, 69% of the quarters infected with bacteria in pulsotype 1 were from primiparous cows and 82% of the quarters infected with bacteria in pulsotype 2 were from primiparous cows. As 7 of the 8 quarters that cured over the dry period had pulsotype 2, the difference in parity representation is explicable via quarters curing over the dry period. However, it reveals the possible confounding effects on PFGE pulsotype. The actual effect of parity on bacterial and SCC counts may have been confounded by the possibility that certain PFGE pulsotypes may be more likely to cure than others, leaving other PFGE pulsotypes over-represented in later lactation cows with infections spanning multiple lactations. Also of interest is that the ATCC Newbould strain was grouped at >80% similarity into PFGE pulsotype 1. While infections due to the Newbould strain have been described as “typically” having only moderate increases in SCC (Studer et al., 2008) and shedding low bacterial numbers, quarters clustered into PFGE pulsotype 1 shed consistently higher levels with a median cfu/0.01mL of 80 and a median LS of 7.2 (>900,000 cells/mL) compared to PFGE pulsotype 2 isolates with a median cfu of 17 cfu/0.01mL and a median LS of 5.0 (400,000 cells/mL). Also of great interest is the fact that, at the cow level, the median monthly composite test-day LS for cows with quarters grouped in pulsotype 2 was 4.2 (<250,000 cells/mL). In fact every cow with a quarter grouped in pulsotype 2, regardless of parity, had a median monthly composite test-day
LS ≤ 4.8 (350,000 cells/mL). As many dairy personnel use monthly composite test-day information as a means to identify cows with IMI, it is evident from the present study that such approaches will be ineffective at identifying SA infected cows, thus limiting the success of SA IMI eradication programs dependent on test–day information.

In evaluating the extreme differences in individual shedding patterns across cows, within cows and even within quarters across sample periods, an important consideration should be made in reflecting on the effect of sample collection and processing. As it is virtually impossible to collect a sterile sample truly representative of the entire milking, it is equally impossible to examine the total volume of milk microbiologically. The fact that 0.01 to 0.1 mL of milk is used to represent the total pounds of milk produced at a single milking might have a significant impact on the perceived “cyclical” variation in bacterial shedding. Therefore, while general trends (high or low median cfu or LS) may be explained by strain relatedness, actual variation may be best explained by sample collection and processing and not by extensive modeling. Given the extent of within cow and between cow differences, future attempts at modeling necessitate factoring in the duration of infection, strain type and potentially immune function. The complexity is best illustrated in cow 4387, which had a quarter (LF) identified at the beginning of lactation with a SA IMI that shed 43% of the time, while a second quarter (LR) began shedding SA in the seventh week of lactation and was culture positive the remaining 27 weeks of the lactation. It is also noteworthy that each quarter in cow 4387 was infected with the same pulsotype (type 1) further highlighting the complexity of differences in shedding patterns and the need for further research aimed at understanding the cause of such dramatic differences within cows.
Although sample variation is inevitable, it highlights the need for consistency for the remainder of all related procedures, including sample collection, sample volume, inoculum volume, and plate counting so that future comparisons are at least contextually possible. Such consistency does not currently exist and due to the perceived intermittent shedding of SA, there have been variable prerequisites applied to screening for and the confirmation of SA IMI. NMC’s published guidelines suggest that 2 of 3 consecutive cultures should be positive for a quarter to be considered infected. (NMC, 1999) Although such requirements may be well suited to research conducted in experimental settings, they are cumbersome and cost prohibitive in field studies and day-to-day mastitis diagnostics. In the present study, the ability to detect SA using microbiological culture was tremendously consistent (95%) when using the larger (0.1mL) inoculum volume. Although shedding varied tremendously in both the number of cfu and shedding pattern, it appeared that such patterns were of little consequence in the accurate detection of SA IMI in these quarters. Erskine reported that percentage agreement between duplicate samples was highest for contagious pathogens such as SA (94.2%), in agreement with the observations from the study of Torres et al., suggesting that a single quarter sample might be adequate in determining infection status. (Erskine & Eberhart, 1988; Torres, Rajala-Schultz, & DeGraves, 2009) Our study examining naturally occurring infections would support the conclusions of Torres and Erskine.

Several limitations are noted in the data that was collected. While Studer et al. identified three shedding patterns amongst 11 quarters using cell equivalents obtained using real-time quantitative PCR, we were unable to identify any predictable shedding pattern of SA using microbiological culture results. Only samples from positive quarters
negative for SA or with a single SA colony were re-examined with a larger volume and it was assumed that samples culture positive at a lower volume would remain positive at a greater volume. As this study was done on only two dairies, results may not be representative of other strains of SA such as the “low shedding” natural infections noted by Sears et al., although as noted (Figure 4.8) the Newbould strain was grouped in to one of our strain types at >80% similarity (at an individual sub-type when grouped using ≥90% similarity). Nevertheless, the present study represents the most exhaustive examination of naturally infected quarters to date. Therefore, much can be gained from this study of naturally occurring SA infections over such an extended sample period. As far back as 1973, a call was made for science to reach an agreement as to what actually defines “mastitis” in terms of cell count or culture. (Neave, 1973) Neave pointed out then that the definitions of mastitis were “not suitable” and “cumbersome” and wrote that the then current definition of “normal” was too rigid. As such an agreement has yet to be accomplished, little if any continuity or comparability between published studies is possible. Although this single study does not answer all of our questions regarding diagnostic criterion for all IMI, it is one of few that have examined naturally occurring SA IMI for an extended period of time and may further aid in developing more coherent prerequisites for the diagnosis of SA IMI.
5.5 References


NMC. 1999. *Laboratory Handbook on Bovine Mastitis* (Revised ed.).


USDA. 2008. Milk Production, Disposition and Income 2007 Summary: USDA.
Table 5.1 Median cfu/0.01mL, linear score (LS), and composite test day LS (Test Day LS) for 29 SA quarters (Cow ID-Qt; right front=RF, right rear=RR, left front=LF, left rear=LR) and the number of samples culture positive for SA (SA +) for each quarter using a 0.01 and 0.1mL inoculum. (LS =4 is equivalent to a SCC of 200,000 cells/mL; LS of 5.3 = 500,000 cells/mL)

<table>
<thead>
<tr>
<th>Cow ID-Qt (# samples)</th>
<th>cfu/0.01mL median (mean)</th>
<th>LS</th>
<th>Test-Day LS</th>
<th>SA+ samples 0.01mL (%)</th>
<th>SA+ samples 0.1mL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70-LR (40)</td>
<td>2 (43)</td>
<td>4.7</td>
<td>4.6</td>
<td>24 (60%)</td>
<td>40 (100%)</td>
</tr>
<tr>
<td>74-LF (26)</td>
<td>1000 (832)</td>
<td>8.9</td>
<td>6.6</td>
<td>26 (100%)</td>
<td>26 (100%)</td>
</tr>
<tr>
<td>101-LF (44)</td>
<td>317 (422)</td>
<td>8.6</td>
<td>5.7</td>
<td>43 (98%)</td>
<td>43 (98%)</td>
</tr>
<tr>
<td>101-RF (44)</td>
<td>1 (25)</td>
<td>4.5</td>
<td>5.7</td>
<td>28 (64%)</td>
<td>37 (84%)</td>
</tr>
<tr>
<td>111-RR (43)</td>
<td>395 (435)</td>
<td>8.7</td>
<td>7.4</td>
<td>42 (98%)</td>
<td>43 (100%)</td>
</tr>
<tr>
<td>115-LR (41)</td>
<td>1000 (749)</td>
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<td>7.3</td>
<td>41 (100%)</td>
<td>41 (100%)</td>
</tr>
<tr>
<td>115-RF (41)</td>
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<td>8.2</td>
<td>7.3</td>
<td>40 (98%)</td>
<td>41 (100%)</td>
</tr>
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<td>115-RR (41)</td>
<td>11 (38)</td>
<td>6.8</td>
<td>7.3</td>
<td>40 (98%)</td>
<td>41 (100%)</td>
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<td>132-LR (37)</td>
<td>47 (128)</td>
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<td>37 (100%)</td>
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<tr>
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<td>3.8</td>
<td>34 (92%)</td>
<td>37 (100%)</td>
</tr>
<tr>
<td>1383-RF (33)</td>
<td>8 (100)</td>
<td>5.3</td>
<td>3.9</td>
<td>27 (82%)</td>
<td>33 (100%)</td>
</tr>
<tr>
<td>1384-LR (39)</td>
<td>329 (501)</td>
<td>8.7</td>
<td>7.3</td>
<td>34 (87%)</td>
<td>39 (100%)</td>
</tr>
<tr>
<td>1384-RR (39)</td>
<td>383 (522)</td>
<td>8.1</td>
<td>7.3</td>
<td>39 (100%)</td>
<td>39 (100%)</td>
</tr>
<tr>
<td>1433-RF (29)</td>
<td>6 (28)</td>
<td>5.3</td>
<td>3.8</td>
<td>26 (90%)</td>
<td>29 (100%)</td>
</tr>
<tr>
<td>1574-LF (28)</td>
<td>97 (231)</td>
<td>5.5</td>
<td>4.5</td>
<td>28 (100%)</td>
<td>28 (100%)</td>
</tr>
<tr>
<td>1574-RF (28)</td>
<td>114 (200)</td>
<td>4.9</td>
<td>4.5</td>
<td>27 (96%)</td>
<td>28 (100%)</td>
</tr>
<tr>
<td>1612-RR (41)</td>
<td>15 (83)</td>
<td>6.4</td>
<td>3.7</td>
<td>39 (95%)</td>
<td>41 (100%)</td>
</tr>
<tr>
<td>1617-LR (34)</td>
<td>0 (30)</td>
<td>4.4</td>
<td>4.8</td>
<td>15 (44%)</td>
<td>26 (77%)</td>
</tr>
<tr>
<td>1617-RR (34)</td>
<td>3 (23)</td>
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<td>4.8</td>
<td>22 (65%)</td>
<td>29 (85%)</td>
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<tr>
<td>1666-RR (27)</td>
<td>56 (70)</td>
<td>5.9</td>
<td>4.6</td>
<td>24 (89%)</td>
<td>26 (96%)</td>
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<tr>
<td>4320-RF (44)</td>
<td>477 (564)</td>
<td>9.0</td>
<td>7.8</td>
<td>44 (100%)</td>
<td>44 (100%)</td>
</tr>
<tr>
<td>4348-LF (30)</td>
<td>15 (54)</td>
<td>7.8</td>
<td>5.1</td>
<td>20 (67%)</td>
<td>28 (93%)</td>
</tr>
<tr>
<td>4387-LF (44)</td>
<td>0 (26)</td>
<td>5.2</td>
<td>4.5</td>
<td>8 (18%)</td>
<td>17 (43%)</td>
</tr>
<tr>
<td>4393-LF (40)</td>
<td>17 (81)</td>
<td>3.2</td>
<td>2.7</td>
<td>29 (73%)</td>
<td>36 (90%)</td>
</tr>
<tr>
<td>4401-LF (37)</td>
<td>70 (106)</td>
<td>6.8</td>
<td>5.4</td>
<td>36 (97%)</td>
<td>37 (100%)</td>
</tr>
<tr>
<td>4401-RF (37)</td>
<td>11 (33)</td>
<td>6.4</td>
<td>5.4</td>
<td>35 (95%)</td>
<td>36 (97%)</td>
</tr>
<tr>
<td>4403-LF (37)</td>
<td>52 (81)</td>
<td>4.6</td>
<td>4.8</td>
<td>35 (95%)</td>
<td>36 (97%)</td>
</tr>
<tr>
<td>4564-LF (38)</td>
<td>244 (360)</td>
<td>6.5</td>
<td>4.7</td>
<td>38 (100%)</td>
<td>38 (100%)</td>
</tr>
<tr>
<td>4729-LF (38)</td>
<td>38 (97)</td>
<td>4.5</td>
<td>3.1</td>
<td>32 (84%)</td>
<td>35 (92%)</td>
</tr>
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</table>
Table 5.2 Mean, median and standard deviation over all quarters and by Parity (lactation) and PFGE pulsotype (PFGE) for milk production (in pounds), bacterial counts (cfu/0.01mL), log transformed cfu (ln-cfu/0.01mL), Linear score (LS), composite test day results (Test Day SCC x1000) and Linear score of monthly composite test-day values (test Day LS).

<table>
<thead>
<tr>
<th>(n= quarters)</th>
<th>Milk (lbs)</th>
<th>CFU 0.01mL</th>
<th>ln(cfu) 0.01mL</th>
<th>LS</th>
<th>Test Day SCC (x1000)</th>
<th>Test Day LS</th>
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<tbody>
<tr>
<td></td>
<td>(n=1064)</td>
<td>(n=1070)</td>
<td>(n=1070)</td>
<td>(n=1049)</td>
<td>(n=187)</td>
<td>(n=187)</td>
</tr>
<tr>
<td>All Quarters</td>
<td>Mean 66.4</td>
<td>215.8</td>
<td>3.4</td>
<td>6.3</td>
<td>1040.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Median 66.7</td>
<td>54</td>
<td>4.0</td>
<td>6.4</td>
<td>460</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>S.D. 21.6</td>
<td>329.8</td>
<td>2.78</td>
<td>2.2</td>
<td>1179.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Lactation</td>
<td>(n=749)</td>
<td>(n=752)</td>
<td>(n=752)</td>
<td>(n=735)</td>
<td>(n=137)</td>
<td>(n=137)</td>
</tr>
<tr>
<td>1</td>
<td>Mean 62.7</td>
<td>164.1</td>
<td>3.1</td>
<td>6.1</td>
<td>936</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>Median 63.4</td>
<td>37</td>
<td>3.6</td>
<td>6.1</td>
<td>460</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>SD 18.9</td>
<td>278.8</td>
<td>2.6</td>
<td>2.1</td>
<td>971</td>
<td>2.2</td>
</tr>
<tr>
<td>≥2</td>
<td>(n=315)</td>
<td>(n=318)</td>
<td>(n=318)</td>
<td>(n=314)</td>
<td>(n=50)</td>
<td>(n=50)</td>
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<tr>
<td></td>
<td>Mean 75.3</td>
<td>337.9</td>
<td>4.0</td>
<td>6.9</td>
<td>1325</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Median 79.2</td>
<td>124</td>
<td>4.8</td>
<td>7.2</td>
<td>476</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>SD 24.7</td>
<td>401.7</td>
<td>2.8</td>
<td>2.2</td>
<td>1597.3</td>
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</tr>
<tr>
<td>PFGE</td>
<td>(n=587)</td>
<td>(n=594)</td>
<td>(n=594)</td>
<td>(n=587)</td>
<td>(n=113)</td>
<td>(n=113)</td>
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<tr>
<td>1</td>
<td>Mean 62.0</td>
<td>267.9</td>
<td>3.8</td>
<td>6.9</td>
<td>1281.5</td>
<td>6.0</td>
</tr>
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<td>82.5</td>
<td>4.4</td>
<td>7.2</td>
<td>985</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>SD 21.8</td>
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<td>2.7</td>
<td>2.0</td>
<td>1051.6</td>
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<td>(n=398)</td>
<td>(n=398)</td>
<td>(n=387)</td>
<td>(n=64)</td>
<td>(n=64)</td>
</tr>
<tr>
<td></td>
<td>Mean 72.1</td>
<td>80.0</td>
<td>2.4</td>
<td>5.1</td>
<td>323.1</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Median 69.5</td>
<td>17</td>
<td>2.8</td>
<td>5.0</td>
<td>222.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>SD 19.3</td>
<td>166.5</td>
<td>2.5</td>
<td>1.9</td>
<td>393.9</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>(n=78)</td>
<td>(n=78)</td>
<td>(n=78)</td>
<td>(n=79)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td></td>
<td>Mean 70.3</td>
<td>511.6</td>
<td>5.2</td>
<td>8.0</td>
<td>2907.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Median 78.9</td>
<td>348</td>
<td>5.9</td>
<td>8.3</td>
<td>1970</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>SD 23.7</td>
<td>428.7</td>
<td>2.3</td>
<td>1.1</td>
<td>2376.53</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Figure 5.1 Lowess smoother graphs for individual SA quarters sampled from 26 to 44 weeks. Band-width of 0.18 is equivalent to an 8 week window. Each graph is identified by the cow ID number and quarter (RF=right front, RR=right rear, LF=left front and LE=left rear). Quarters grouped by boxes are from the same cow. Reference line at 0cfu/0.01mL.
Figure 5.2 Lowess Smoother at over 8 week periods for each quarter of cow 1384 (PFGE pulsotype 3). Each sub-graph is labeled with cow id and quarter.

Figure 5.3 Weekly shedding (cfu/0.01mL) of SA for Cow 101 in both quarters, left-front (LF) and right-front (RF) showing the difference in shedding within cows.
Figure 5.4 Weekly shedding (cfu/0.01mL) of SA for Cow 115 in three quarters, left-rear (LR), right-front (RF) and right-rear (RR) showing the difference in shedding between 3 quarters in the same cow.
Figure 5.5 PFGE from representative isolates from each SA quarter sampled ≥ 26 weeks in addition to isolates from quarters that cured (-C) over the dry period. Primary clusters were defined as those with >80 similarity, labeled numerically (1, 2 and 3).
Figure 5.6  Lowess Smoother panels over 8 week periods for each PFGE pulsotype, 1 and 2. Each sub-graph in each panel is labeled with cow id and quarter, with reference line on y-axis placed at 0 cfu/0.01mL equivalent.
Figure 5.7. Histogram of the frequency of monthly composite test-day SCC expressed as a linear score by PFGE pulsotype designated numerically in separate graphs 1-3. Reference line on x-axis placed at 500,000 cell/mL equivalent.
CHAPTER 6

THE EFFECT OF STRAIN TYPE ON CURE-RATES IN DAIRY COWS WITH NATURALLY OCCURRING STAPHYLOCOCCUS AUREUS INTRAMAMMARY INFECTIONS

6.1 Introduction

Despite improvements in management, prevention, treatment and vaccines, mastitis continues to be one of the most prevalent and costly diseases in dairy cattle. (USDA, 2007) The estimated annual cost of mastitis to the dairy industry is 2.1 billion dollars, 70-80% of which is due to subclinical intramammary infections (IMI) such as those caused by Staphylococcus aureus (SA). (Bar et al., 2008; Shim, Shanks, & Morin, 2004) At a prevalence of 43%, SA is the most common of all contagious pathogens on US dairies. (USDA, 2007) The high prevalence and dramatic economic impact of SA continue to motivate interest in developing effective treatments and better strategies for managing SA IMI.

Although initial perceptions of the efficacy of intramammary antibiotics were quite positive, few producers or veterinarians today would agree with such predictions. (Erskine, Wagner, & DeGraves, 2003; Hoe & Ruegg, 2005) Treatment outcomes are dependent on multiple factors including the host, pathogen, treatment and duration of both the infection and therapy. A single reason for a treatment failure is therefore difficult to identify. A meta-analysis evaluating treatment of subclinical SA
mastitis found that minimum inhibitory concentrations (MIC) were of no use in predicting bacteriologic cure. (Constable & Morin, 2003) Due to the poor performance and predictability of approved therapies, new therapeutic approaches have been developed. Erskine et al. reported that if the initial therapy using the approved label dose proved ineffective, the best approach was to extend that therapy rather than to switch drugs or increase the dosage. (Erskine, Wagner, & DeGraves, 2003) Previously unavailable in the U.S., such therapies are now possible due to new product labels allowing for extended treatment without interruption.

The lack of predictability of cure based on antimicrobial profiles has also inspired the application of DNA genotyping to study the association between SA strains and probability of cure. Comparing PFGE patterns of clinical isolates, Haveri et al. found an association between pulsortype, the severity of infection and probability of cure. (Haveri, Taponen, Vuopio-Varkila, Salmenlinna, & Pyorala, 2005) The application of molecular technology may provide information that is useful in making better treatment and culling decisions of SA cows. The ability to use PFGE to also identify subclinical infections with a greater probability of cure would allow producers to selectively treat and cull SA cows, thereby encouraging the judicious use of antibiotics while improving management and decision making. The objective of this study was to compare treatment outcomes of extended intramammary therapy between strains of SA as determined by PFGE. We hypothesized that strain differences would account for variability in response to therapy.

6.2 Materials and Methods

6.2.1 Study Population and Sampling
Eleven cows identified with subclinical SA IMI during a whole herd sampling and an additional 15 cows identified at freshening in a single herd were enrolled in the study. During this initial screen, composite milk samples were collected according to National Mastitis Council (NMC) guidelines. (NMC, 2004) Quarters with grossly abnormal milk were sampled separately. Samples were placed on ice for transport and frozen immediately upon return to the laboratory. The following day, 0.01 ml of milk was plated on washed cow blood agar with 0.1% Esulin (Hardy Diagnostics). Plates were incubated at 37°C and screened at 24 and 48 hours according to NMC guidelines. Every quarter of each cow identified as SA positive on composite samples was sampled the following week to identify the infected quarter. One sample from each quarter was collected for culture and frozen while a second sample was collected and preserved for somatic cell count (SCC) evaluation. A second consecutive sample of each SA quarter was collected one week later to confirm the presence of SA infection. Thus, each quarter enrolled in the study cultured positive for SA (≥100 cfu/ml) at least twice prior to treatment. Each cow was treated once a day with an approved lactating cow treatment (pirlimycin hydrochloride) for a total of six days. Following treatment, each quarter was sampled weekly to assess cure-rates. Milk samples were cultured for SA and analyzed for SCC until they were found to be shedding SA again (treatment failure) or until the end of their lactation or the trial. Cure was defined as bacteriologic, that is, any cow culturing positive for SA at any point after treatment was considered a treatment failure.

6.2.2 Strain Comparisons
Pre- and post-treatment SA isolates were analyzed using PFGE at The Ohio State University, Infectious Disease Molecular Epidemiology Laboratory (IDMEL). The PFGE protocol used by IDMEL is adapted from the Canadian protocol created by a subcommittee of The Canadian Committee for the Standardization of Molecular Methods (CCSMM) involving a team lead by Mulvey et al. (Mulvey et al., 2001). A dendrogram of SA isolates was developed using Bionumerics software version 4.6 (Applied Maths, Austin, TX). Isolates were grouped by cluster analysis based on the number and position of the bands. Isolates with three or fewer band differences were considered subtypes of a single strain, while isolates with greater than three band differences were counted as separate strains (Tenover et al., 1995) resulting in clusters with >90% similarity.

6.2.4 Antibiotic Profiling

Minimum inhibitory concentrations (MIC) of pre- and post-treatment SA isolates for ten different antimicrobials were determined using the Trek Diagnostic Sensititre system (Cleveland, Ohio).

6.2.5 Statistical Analysis

In addition to evaluating cure based on PFGE pulsotype, association of cure with parity, SCC and last composite test-day SCC was evaluated using Fisher’s Exact test. Cows were categorized based on composite test day SCC and SCC of infected quarters to study the association between SCC and cure. SA quarters with SCC < 250,000 on the first day of treatment were considered to have a low SCC; SA quarters with SCC ≥ 250,000 were considered to have a high SCC. The cutoff between low and high SCC cows using
test day composite sample was 500,000 SCC/ml. Descriptive statistics and comparisons were evaluated using STATA v.10 (STATA Corp, College Station, Texas).

6.3 Results

Of the 26 cows enrolled in the study, one was dropped due to illness while three were dropped due to inappropriate administration of the treatment protocol. In total, 12 first lactation heifers and 10 second or greater lactation cows were included in the analysis. Descriptive summary statistics are presented in Table 1. A total of six (27%) quarters were cured, testing negative for the remainder of the sampling period (14 -32 weeks). Of the failed quarters, 12 cultured positive the first week following treatment and 4 were culture negative for at least 1 week. A total of nine different genotypic clusters (A-I) were identified among 22 pre-treatment isolates. Cures occurred in five different clusters, two occurring in cluster C containing 8 isolates (Figure 1). All isolates were pansusceptible to the 10 tested antibiotics and remained susceptible post treatment.

Evaluating cure by parity, nearly 42% of first lactation cows cured while only 10% of multiparous cows cured (Fisher’s Exact Test p=0.119). Examination of cure by quarter level SCC revealed that one of three (33.3%) quarters with low SCC (<200,000) cured while five of nineteen (26.3%) high quarters cured (Fisher’s Exact p=0.636). Using the most recent composite test day SCC prior to treatment, five of fifteen (33.3%) low cows (<500,000) cured while only one of seven (14%) high cows cured (Fisher’s Exact p=0.349).
6.4 Discussion

The primary interest of the study was to evaluate the effect of strain on treatment outcome. In addition we examined the effects of parity and somatic cell counts at the quarter and cow level. Six cures occurred in five separate strains. Therefore, a predilection for cure could not be attributed to a specific strain. This may reflect management practices of this herd which had a history of purchasing cows from several sources in addition to outsourcing the rearing of young-stock. Therefore, the results may not be generalizable to the dairy industry at large. Diagnostics such as strain typing may have limited value in developing treatment strategies in herds where strain variability may be substantial. Several reports describing the molecular diversity of SA within farms have been published. Regardless of methods the majority have found that SA IMI isolates from a single farm represent clonal populations, having a predominant strain type with little strain diversity. (Anderson, Lyman, Bodeis-Jones, & White, 2006) Middleton et al. recently examined the diversity of SA in herds dependent on imports for either replacements or expansion. (Middleton, Fox, Gay, Tyler, & Besser, 2002) In that study, PFGE revealed 82% of the strain types were unique to one herd, but herds purchasing replacement heifers had more strains than closed herds.

While several studies have been done examining cure-rates in SA IMI, many have administered therapies not approved in the United States; therefore, application of the findings in the U.S. has been limited. The overall cure-rate with uninterrupted extended therapy in this study was 27%. This is far lower than what has been reported with shorter treatment duration. Deluyker et al. compared extra-label treatment of subclinical cases of SA with the same product used in this study and found cure rates significantly higher with
extended therapy (86%, eight days) when compared to standard therapy (56%, two days) and control (6%, no treatments). (Deluyker, Van Oye, & Boucher, 2005) However, an important consideration is that the comparison was made across two herds, one herd using two-day treatment and the other eight-day treatment. This comparison ignores the potential influences of strain and other herd specific factors on cure-rates.

In a study examining cure rates of clinical infections following dry cow therapy, Dingwell et al. used pulse field gel electrophoresis (PFGE) comparing strains among 40 herds and reported that a lineage group was significantly associated with the probability of cure of subclinical SA. (Dingwell, Leslie, Sabour, Lepp, & Pacan, 2006) However, as lineages with fewer than three isolates were not included in the evaluation of the pulsotypes, the study did not address potential differences in cure rates amongst minor strains. Haveri et al. also found an association between pulsotype, the severity of infection and probability of cure in clinical SA IMI’s. (Haveri, Taponen, Vuopio-Varkila, Salmenlinna, & Pyorala, 2005) The exclusion of subclinical infections in that study introduces a limitation as results would not reflect the majority of SA infections which remain subclinical. Thus the question remains whether technology such as PFGE may be useful to herds developing targeted treatment strategies.

When evaluating the cure-rates in this study, one may consider that a strict definition of cure was applied (bacteriologically negative for the remainder of lactation, minimum of 14 weeks). With no standard definition of cure across mastitis research, some studies have required one negative sample and others have evaluated quarters 4 to 12 weeks after treatment. If a less stringent requirement had been applied, up to 3 additional quarters may have been considered in the cured group. However, as the 3
additional isolates were not in any strain previously associated with a cure, including them would have only further supported our conclusion that likelihood of cure was not associated with a particular strain.

While limited numbers made significance difficult to achieve, our results in comparing cure rates across parity and composite somatic cell counts agreed with previous studies revealing that primiparous cows and cows with composite SCC less than 500,000 are more likely to cure than cows with previous composite SCC ≥500,000. Some investigators have proposed this may be due to the duration of infection often correlating duration with parity. However, it is important to consider that the actual duration of SA infections have never been established microbiologically in any study. Assumptions of chronicity have been made based on parity, stage of lactation and composite test day information. The extent of the influence of chronicity remains unclear as assumptions discount the fact that multiparous cows have likely been treated with standard dry cow therapy, thereby leaving strains less likely to cure in the pool of multiparous study subjects. Interestingly though, in the current study, cure-rates did not appear to be associated with SCC at the quarter level. Such information highlights the need for continued research into the molecular epidemiology of SA mastitis as there appears to be vast differences in the impact of infection on the mammary gland at both the cow and quarter level.
6.5 References


Table 6.1 Days in Milk and Somatic Cell Counts of SA quarters and cows

<table>
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<th>Variable</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
</tr>
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<tbody>
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<td>44</td>
<td>380</td>
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<tr>
<td>SA Quarter SCC (x1000)</td>
<td>2,900</td>
<td>148</td>
<td>9,999</td>
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<td>Composite (Last Test Day) SCC (x1000)</td>
<td>828</td>
<td>100</td>
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<tr>
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<td>Last Test Day Linear Score</td>
<td>5.03</td>
<td>3</td>
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</table>
Figure 6.1 Dendrogram of SA isolates – Boxed isolates represent cured quarters, brackets indicate pulsotypes (isolates with 3 or fewer band differences resulting in clusters of isolates with greater than 88% similarity).
7.1 Major Findings of My Current Research

“I am not here to give you dogma. A dogma makes one certain.”

Bhagwan Shree Rajneesh

The cumulative understanding I have gained from both review of the published literature and my research, in the context of extensive practice experience, has revealed nothing more than further uncertainly in our fundamental understanding of mastitis and its epidemiology, particularly that caused by Staphylococcus aureus (SA). To follow are what I consider to be the most salient points gleaned from my research of SA intramammary infections (IMI) in addition to important question that emerged and remain unanswered. Although none of the proceeding findings are proclaimed with any certainly, they are made with thoughtful reflection in the context of all that I have gathered before me.
7.2 The Effects of Inoculum Volume on the Microbiological Detection of Naturally Occurring *Staphylococcus aureus* Intramammary Infections

- When evaluating the ability of microbiologic culture to detect SA in quarter with a known SA IMI, the relative importance of diagnostic inoculum was varied with the study population and sample schedule.

- Examining daily samples from a single dairy using a 0.01mL inoculum the sensitivity and specificity of microbiologic culture were 97.7% and 100% respectively. Using a larger (0.1mL) inoculum, the sensitivity increased to 99.8% and the specificity was not changed.

- Examining weekly samples from 2 dairies (one of which was included in the daily sample study) using 0.01mL inoculum, the sensitivity and specificity of microbiologic culture was 83% and 98%, respectively. Using a larger (0.1mL) inoculum, the sensitivity increased to 94% and the specificity decreased to 97.6%.

- Combining the results of both studies, we found that the sensitivity of microbiologic culture for detecting SA IMI using a 0.01mL inoculum was 91% with a specificity of 99.4%. Using a larger (0.1mL) inoculum, the sensitivity increased to 96.8% and the specificity was 99.3%.
• This study demonstrated a clear benefit of using a larger inoculum volume (0.1mL) for culture in the microbiologic detection of SA IMI.

• This study also highlighted the lack of consistency pervasive in mastitis research, whether in defining an IMI itself or in defining what and how much is subject to examination. Such inconsistency allows for little comparison across published literature and continues to limit the progression of a true understanding of naturally occurring IMI, particularly that of SA.

7.3 Variation in Daily Shedding Pattern of *Staphylococcus aureus* and Somatic Cell Counts in Naturally Occurring Intramammary Infections

• This study demonstrated that the variability in recovery of SA in samples collected from quarters with a known SA IMI has little consequence in our ability to accurately detect a SA IMI. Although the amount of SA recovered from infected quarters varied tremendously, 97.7% of the samples were culture positive.

• While some have documented specific patterns of shedding and relationships between bacterial counts and somatic cell counts (SCC), we were not able to demonstrate any such pattern or relationship with any consistency. Trends were examined over a 6.5 day period revealing a variety of patterns each with variable amplitudes and duration.
• Examination of daily changes in SA and SCC recovered from SA infected quarters did demonstrate a remarkable independence in values between cows as well as within cows.

• Pulse field gel electrophoresis was used to evaluate the association of strain type and the recovery of SA from infected quarters. The 9 isolates examined were placed into 2 separate groups (at <80% similarity) with only 2 isolates occurring in PFGE pulsotype 2. While quarters infected with SA grouped into the PFGE pulsotype 1 appeared to shed at consistently higher levels and with comparatively little variability, (median cfu/0.01mL=100), recovery of SA from quarters infected with SA placed in PFGE pulsotype 2 was significantly lower (median cfu/0.01mL=59, p < 0.0001)

• As PFGE pulsotype 2 was represented by only 2 of the 9 quarters, conclusions should be made with caution. Further study is certainly warranted to further explore possible influences on shedding patterns of SA IMI and our ability to detect them.

7.4 Shedding Patterns of naturally occurring Staphylococcus aureus intramammary infections sampled weekly throughout lactation
• This study examining naturally occurring SA IM on 2 different dairies further demonstrated the ability to consistently recover SA from naturally infected SA quarters provided a large enough (0.1mL) inoculum is used. Using a larger inoculum, a sensitivity of microbiologic testing of 95% can be achieved, compared to 85% when using a smaller volume (0.01mL).

• As with the study examining daily patterns of recovery of SA from quarter milk samples, this study examining quarters weekly over the entire lactation was also unable to define any specific and consistent shedding pattern, other than that of consistently higher versus consistently lower shedding quarters, either of which was consistently detectable microbiologically.

• Quarters from which consistently higher levels of SA were recovered were again grouped into the same PFGE pulsotype as those from the daily study, further supporting the possible influence of strain type on either persistence or pathogenicity of IMI. Strains clustered into the PFGE pulsotype 1 had a median cfu/0.01mL of 83, while strains grouped into PFGE pulsotype 2 had a median cfu/0.01mL of 17.

• As it has yet been possible to confirm or pinpoint exactly when a quarter becomes infected with SA, it impossible to accurately define the effect of IMI duration on the amount of bacteria or SCC shed in the milk. For this reason further research is
necessary to better understand the epidemiology of the transmission and infection of SA IMI.

- However, in order for such work to be effectual, it is critical that standards be set for sample collection, sample volume examined, and definitions of IMI based on specific pathogens. Until this is done, further comparisons will remain contextually impossible and therefore pragmatically useless.

7.5 The Effect of Strain Type on Cure-rates in Dairy Cows with Naturally Occurring Staphylococcus aureus Intramammary Infections

- This study followed the treatment success of extended intramammary therapy in 22 naturally occurring SA IMI.
- Evaluating cure by parity, 42% of the first lactation cows cured, while only 10% of the multiparous cows cured.
- While much has been made of the increased likelihood of primiparous cows to effectively cure, it should be expected as we have biased the multiparous cows toward treatment failure, given that the vast majority had been subjected to blanket dry cow therapy, thereby leaving infection less amenable to cure in the multiparous cow pool.
Unlike other published works, we found no significant difference (p=0.64) in cure rates in quarters with low quarter level SCC (200,000 cells/mL) and high SCC. Nor was there any significant difference (p=0.35) when evaluating cure using composite test day information.


8. Buelow, K. L. G., William J.1; Collins, Michael T.2; Clayton, Murray K.3; Nordlund, Kenneth V.1; Thomas, Chester B. 1996. A model to determine sampling strategies and milk inoculum volume for detection of intramammary


Appendix A: Assessing Agreement of DeLaval Direct Cell Counter with Somacount 300 on Quarter Milk Samples from Dairy Cows.

The objective of this study was to determine the agreement of the DeLaval Direct Cell Counter (DCC) with the Somacount 300 (DHI Cooperative, Inc) in reporting somatic cell counts (SCC). Over a period of 14 days, quarter milk samples (n= 500) were taken from sixteen cows ranging from 42 to 145 days in milk at a single farm. Two samples, one for DHI evaluation and the second for DCC and culture, were collected from each quarter prior to the evening milking in accordance with National Mastitis Council recommendations. Samples for DCC were evaluated immediately after milking in the laboratory while samples for Somacount evaluation were refrigerated with preservative for 1 to 4 days prior to evaluation. Samples excluded from analysis included those with measurement errors (n=8) and DHI values less than 10,000 (n=129) or greater than 4 million (n=63) SCC.

Although there was reasonably good correlation (91%) between lnSCC and lnDCC, Bland and Altman (BA) analysis revealed poor agreement within the published working range provided by DeLaval for the DCC. For this study limits of agreement were set at 100,000 cells/ml, that is, one could reasonably allow the measurements to be as much as 100,000 cell/ml apart and not limit the clinical application of the DCC test. Limits of Agreement determined by BA revealed that you may expect the DCC value to be as
much as 545,000 cells below to 787,000 cells above that reported by DHI with a mean difference of 121,000. This study is contrary to others that have employed methods of correlation in comparing these two tests. Our findings indicate that, when evaluating the two tests strictly on agreement, there is insufficient agreement for the tests to be used interchangeably. However, from a clinical aspect, when records were examined on a case by case basis only 9% of the samples would have proved contrary in clinical interpretation when subclinical mastitis is defined as >200,000 cell/ml). Nonetheless, 32% of the samples differed by more than 100,000 cells/mL.