ISOLATION AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* FROM BULK TANK MILK FROM MINNESOTA DAIRY FARMS

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Dedication

This dissertation is dedicated to my beloved parents and brother for their continued moral support, guidance and care.

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Chapter 1

Introduction

Bovine mastitis is a significant disease of dairy cattle worldwide. *Staphylococcus aureus*, an opportunistic pathogen of humans and animals, is a major cause of mastitis in dairy farms (101). In order to combat mammary infections, antibiotics are administered in therapeutic doses for treatment and prevention of the disease (37). Thus bovine mastitis is a reason for antibiotic use on dairy farms.

The projected annual losses due to mastitis are about 2 billion dollars annually. Emergence of antibiotic resistant *S. aureus* would lead to increase in length and cost of treatment of *S. aureus* mastitis.

It has been observed that *S. aureus* has great adaptive power to antimicrobial agents. This has been observed in the emergence of penicillin resistant *S. aureus* and methicillin resistant *S. aureus* (MRSA) following the introduction of β-lactam antibiotics (6).

In addition to the traditional hospital and community reservoirs of MRSA, a large reservoir in farmed animals has been identified and labeled as livestock associated MRSA (102).

Very few studies in the United States have reported farm level prevalence of MRSA in dairy operations. Few reports in the United States describe prevalence rates of 0-1.8% for the presence of MRSA in milk samples from Michigan, Wisconsin, North Carolina, Virginia and other states in the U.S (8, 33, 59, 99).

Objective: This study was designed to identify the prevalence of *S. aureus*

including methicillin resistant *S. aureus* (MRSA) in dairy farms of Minnesota.

Specific Aims:

1) To describe the prevalence and distribution of Methicillin susceptible *S. aureus* (MSSA) and MRSA in bulk tank milk (BTM) samples from MN dairy farms.

2) Describe the genotypic and phenotypic diversity among MSSA and MRSA isolates using MLST, PFGE and antibiotic susceptibility testing, respectively.

3) Identify farm-level risk factors associated with the presence of multidrug resistant *S. aureus* and MRSA in MN dairy operations.

Chapter 2

Review of Literature

Bovine mastitis

Bovine mastitis (inflammation of the mammary gland) is a major disease of dairy cattle worldwide. Mastitis is associated with complex etiology - multiple microorganisms are implicated but it is usually bacteria that invade the teat cistern and produce a multitude of virulence factors that lead to inflammation and tissue damage. Bovine mastitis can be classified into three categories based on the severity of the infection: subclinical, clinical and chronic forms. The severity of infection is dependent on the nature of the causative organism, and on the age, breed, health status, and lactation state of the animal (98).

Clinical mastitis is manifested with local and sometimes generalized clinical signs such as heat, pain and swelling of infected udder. It also results in milk abnormalities such as flakes, clots or watery secretions (37). Subclinical mastitis is characterized by milk production losses and lowered milk quality (37, 98). Chronic mastitis occurs less frequently and is characterized by persistent inflammation of the mammary gland. Clinical mastitis can result in agalactia of infected quarters leading to premature culling and can be fatal in acute cases (37). Subclinical mastitis on the other hand does not pose an urgent threat to the cow's life; the main effect is an increase in somatic cell counts (SCC) which leads to financial losses to the farmer (37). Economic losses associated with both forms of the disease are due to poor milk quality, less milk production, early culling of cows and increased treatment and labor costs to the farmer (98).

In the United States alone, the projected losses due to mastitis are 2 billion dollars annually (16, 70, 78). About 70-80% of the estimated $ 140-300 loss per cow/year caused by mastitis to the dairy industry is due to reduced milk production from asymptomatic mastitis (90).

Bacteria are the primary cause of mastitis; however mastitis caused by viruses, fungi and algae has also been reported (78). Mastitis can be segregated into contagious and environmental mastitis based on the ecology of the causative organism.

Contagious mastitis causes intra mammary infections (IMI) transmitted from one cow to another, with the primary reservoir of such pathogens being infected quarters (35). Spread of the contagious pathogen to uninfected udders occurs during the milking process. The bacteria responsible for contagious IMI include streptococci (*Streptococcus agalactiae, Streptococcus dysgalactiae*), coagulase positive staphylococci (CPS or *Staphylococcus aureus*) and *Mycoplasma bovis*. In mastitis diagnostics, staphylococci are divided into CPS and CNS based on their ability to coagulate rabbit plasma (92). The major pathogen *S. aureus* is generally coagulase positive. However, more than 10 different coagulase negative staphylococci have been isolated from mastitic bovine milk and the most commonly reported are *Staphylococcus chromogenes* and *Staphylococcus simulans.* In addition, *S. hyicus* and *S. epidermidis* have also been frequently isolated (92). However, CNS are normally not identified to the species level in routine mastitis diagnostics but treated as one uniform group. Other CNS species commonly reported from cow's bedding and environment include *S. xylosus, S. sciuri, S. saprophyticus* (60). Many other species are reported from cow's skin such as *S. chromogenes, S. warnerii, S.*

epidermidis (92).

Environmental mastitis is caused by pathogens present in the dairy barn and in the cow's environment including the dirt, mud, manure, cattle yards and cattle pastures (88). Infection may occur at any time during the cow's life including at milking, between milkings, and during the dry period. The primary causative microorganisms include gram negative bacteria, mainly Enterobacteriaceae with *Escherichia coli, Klebsiella sp., Enterobacter sp., Serratia sp., Proteus sp*., and Pseudomonadaceae. They also include gram positive organisms such as the environmental streptococci (mainly *Streptococcus uberis* and less frequently *Streptococcus dysgalactiae*) (37). Environmental mastitis is primarily associated with clinical mastitis rather than subclinical infection (37).

The most frequently identified pathogens in clinical mastitis include Enterobacteriaceae (primarily *E.coli*), *S. aureus* and *S. uberis* (10). *S. aureus* mastitis is difficult to treat as it often results in deep-seated abscesses. Also, the ability of this microorganism to survive inside phagocytic cells (where antibiotic concentrations are lower), its resistance to multiple antibiotics, particularly β- lactams, and its ability to produce exotoxins which harm the cow's udder result in low cure rates and frequent relapses for *S. aureus* mastitis treatment (37).

Due to high treatment costs and financial losses associated with the disease, early diagnosis is of utmost importance. Diagnostic methods involve detection of mammary gland inflammation as well as detection and characterization of the infecting organism. Detection of the infection is carried out by a number of methods which include measurement of SCC's, enzymatic assays measuring concentrations of elevated enzymes

in milk (e.g. NAGase or LDH) and' cow-side' tests such as the California Mastitis Test (CMT) (78, 83). Mastitis diagnostic tests which involve culturing of microorganisms from milk or other samples is still the gold standard for confirmation of infection and to characterize the mastitis-causing pathogens (98).

Mastitis Prevention and Treatment

Current efforts in mastitis control focus both on prevention and treatment. Preventive measures constitute infusion of intra-mammary (IMM) antibiotics during the dry period. An internal teat sealant can also be used to help prevent new infections during drying off period (106). Other measures to prevent spread of IMI's include pre-milking and post-milking teat antisepsis. This method involves dipping or spraying an antiseptic on the teat surface both before and after milking (12, 13). Treatment for clinical and subclinical mastitis infections involves administration of antibiotics either via the intramammary route (to achieve higher concentration in the teat cistern) or parenterally (to ensure antibiotic infusion into the mammary gland via the mucous membrane) (62).

Treatment in lactating cows:

Treatments to cure mastitis in lactating cows involve both intra-cisternal and, in the case of severe toxic clinical mastitis cases, parenteral route of antibiotic infusion (110). Concomitant use of both routes has been reported to show increased efficacy (110). Compared to the parenteral route of administration, the intramammary route enables very high levels of antibiotics delivered directly into the udder, thus allowing for smaller amount of antimicrobial use and extended activity time (37). Successful treatment involves clearing of the infection from the affected quarter.

Subclinical mastitis infections are generally not treated during the lactating period because of low cure rates and economic costs associated with treatment and withdrawal period for milk (37, 107). Despite these, some techniques for treating subclinical mastitis include – "blitz" and parallel treatment (37). "Blitz" involves treating the entire herd with an intramammary infusion of an antibiotic that the organism has been found to be susceptible, and historically has been used to address herds with a high prevalence of infection with *Strep. agalactiae*. Parallel treatment involves treating subclinically infected quarters together with clinically infected quarters with the same antibiotic.

Treatment at drying off:

Treatment at dry off is an important means of control for both clinical mastitis and subclinical mastits during the non-lactating period. Dry cow therapy (DCT) has a dual effect of: treating IMI's present at the time of dry off and in the prevention of new infections at the beginning of the dry period. Both parenteral and IMM infusion of antibiotic can be carried out as there is no concern about withdrawal periods or antibiotic residues in milk (15). If antibiotics are infused after the last milking of lactation, they remain in the mammary gland in high concentrations for longer periods of time as they are not milked out (15). DCT is reported to have lower cure rates for *S. aureus* mastitis as compared to streptococcal mastitis with 50-80% rates compared to 75-99% respectively (71, 77).

In summary, mastitis is a major cause of therapeutic and sub-therapeutic use of antimicrobial agents on dairy farms and *S. aureus* is significant cause of mastitis in dairy cows as described in the following sections (101).

The organism: Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium belonging to the family *Staphylococcaceae*. These are spherical bacteria which occur in microscopic clusters resembling grapes. *S. aureus* is a common opportunistic pathogen of both humans and animals. In humans, *S. aureus* is the most common cause of nosocomial episodes in humans including skin and soft tissues (SSTI) and post-surgical infections. It can be found on different parts of the body but the most common carriage site is the anterior nares (19). *S. aureus* is known to cause a variety of infections in humans which can be classified as three general types i) superficial skin lesions ii) systemic and life threatening conditions such as endocarditis, osteomyletis, pneumonia, brain abscesses, meningitis and bacteremia and iii) toxinoses such as food poisoning, scalded skin syndrome and toxic shock syndrome (6). As mentioned previously, it is also a significant cause of bovine mastitis in dairy cows.

S. aureus has a tendency to rapidly acquire antibiotic resistance to different classes of antibiotics. With the discovery of penicillin by Flemming in 1929, use of penicillin became widespread in the 1940's during World War II when production costs were lowered and it was mass-produced. However, by the 1950's, *S. aureus* resistant to penicillin emerged which carried plasmids capable of synthesizing penicillinase, or conferred resistance to penicillin. Consequently, when synthetic penicillins were designed to counter this problem and methicillin (a synthetic β-lactam antibiotic) was introduced in 1959, methicillin resistant strains of the bacterium were reported within a year of its use in U.K (38).

mecA -

Methicillin resistant *Staphylococcus aureus* or MRSA was thus 'born' in the 1960's when it acquired *mecA*, a 2.1 kb exogenous gene fragment by horizontal gene transfer. The *mecA* encodes a modified penicillin binding protein, PBP 2a, which has very low affinity for β-lactam antibiotics (40). In the presence of β-lactam antibiotics, the PBP2a transpeptidase assisted by the transglycosidase domain of the native PBP 2 of *S. aureus*, compensates for the cell wall biosynthesis function in the cell (74).

The origin of the *mecA* in *S. aureus* is not clear. A homolog of *mecA* with significant sequence similarity to that carried by *S. aureus* has been identified in *S. sciuri* and was found ubiquitously in the antibiotic susceptible species. It has been postulated that *mecA* could have been acquired by horizontal transfer of the gene from *S. epidermidis* or *S. sciuri* (103).

The staphylococcal cassette chromosome mec (*SCC*mec)

The SCC*mec* is the mobile genetic element that carries *mecA* in the *S. aureus* genome. The SCC*mec* contains two specific recombinase genes designated, cassette chromosome A (*ccrA*) and cassette chromosome B (*ccrB*) as well as different transposons and integrated copies of plasmids which confer resistance against non-β lactam antibiotics (49). This is one reason why MRSA often accumulate resistance to multiple other antibiotics in addition to β-lactam antibiotics. To date five different kinds of SCC*mec* have been described based on the class of *ccr* and the type of *mecA* present. SCC*mec* typing is carried out using specified primer sets to identify the *ccr* and *mec* variants present and classify them as types I-V (108). The detection of different SCC*mec*

types and the presence of divergent MRSA lineages within a MRSA genotype, suggests that MRSA has arisen by multiple independent introductions of *mecA* into successful MRSA lineages (6).

Emergence of distinct MRSA genotypes

Healthcare Associated MRSA

By the 1970's healthcare associated MRSA (HA MRSA) was endemic in many US hospitals and primarily affected the elderly and chronically ill patients (5). Risk factors for MRSA acquisition included hospital care, care in chronic facilities and nursing homes for elderly people, presence of indwelling devices or chronic wounds and previous antibiotic treatment (39). Such infections were difficult to treat as the organisms were resistant to most of the other commonly used antibiotics (39). The prevalence of MRSA in hospitals continues to increase worldwide. The National Nosocomial Infection Surveillance in the US reported that 51% of *S. aureus* isolates from 1998-2002 were methicillin resistant, a 25% relative increase from rates reported for 1995-99 (6). The prevalence of MRSA in central Europe increased from 1.7% in 1990 to 8.7% in 1995 (104). The isolates from HA MRSA infections usually carry a pulsed field gel electrophoresis (PFGE) fingerprint type of USA 100, USA 500 or USA 800 and majority of these do not carry genes for the Panton Valentine Leukocidin (PVL) toxin producing gene (61). The majority of HA MRSA strains are classified into common clonal complexes (CC) as defined by multi locus sequence typing (MLST) in Europe: CC5, CC8, CC22, CC30 and CC45(28).

Community Associated MRSA

Around the late 1990's, community associated MRSA (CA MRSA) emerged in young and healthy people without the typical hospital connections. Since then, there has been a rapid emergence of CA MRSA first in Australia and then in the USA where the target populations constitute underprivileged aboriginal communities, school children, prison inmates, soldiers, athletes and men who have sex with men (93). Risk factors for the development of CA MRSA infection include close contact with people, living in crowded facilities, poor hygiene, sharing of personal items, and contact sports (30, 31). CA MRSA is mainly known to cause skin and soft tissue infections. Serious invasive infections like necrotizing pneumonia associated with a lethality of 75% are also reported to be caused by CA MRSA (36).

In the United States, a particular clone associated with PFGE profile USA 300 and MLST ST 8/SCC*mec* IV clone, harboring the *lukS-lukF* encoding the PVL toxin has led to an epidemic rise in CA MRSA infections (93). USA types 300 and 400 with SCC*mec* IV or V have frequently been isolated from CA MRSA (61).

Livestock associated MRSA

In the past six years, livestock have been identified as a potential reservoir and vector for the transmission of MRSA to humans. The first reports of widespread colonization of pigs and pig farmers were in the Netherlands. Dutch pig farmers had a colonization rate 760 times that of the general Dutch population (100). Since then intensive studies have been conducted in pigs, poultry, and cattle in Europe as well as in the U.S (25, 50, 89, 105). The European Food Safety Association (EFSA) has identified

MRSA in pig holdings in seventeen EU member states (2). The MRSA clone isolated from a majority of these pigs was non-typeable by PFGE after *Sma*I restriction digestion (due to a DNA methylation at the restriction site (11)), was tetracycline resistant and belonged to MLST CC 398 (2). CC 398 has also been detected in other animals such as cattle (2) and poultry (65). Although most MRSA remain colonized on the animals, some cases of infections due to MRSA have been reported in pigs (84) and horses (21).

MRSA in cattle

MRSA was first reported from mastitic milk in dairy cattle in 1972 (29). Since then occasional cases of MRSA mastitis at a low prevalence have been isolated from *S. aureus* isolates (34, 42, 48, 96). Recent reports in Europe indicate prevalence rates of MRSA in bovine milk in the range of - 1.4% in Switzerland (42), 1.5% in Japan (41), 2.4% in Korea (63), 0-7.4% in Belgium (96) and 5.1-16.7% in Southwest Germany (91). Similarly, few studies in the United States have reported zero to low occurrence of MRSA among *S. aureus* isolates from bovine milk in Michigan (33), Wisconsin (59) and North Carolina and Virginia (8). However, these studies report MRSA from routine diagnostic testing of lesions or milk samples for *S. aureus* isolates for phenotypic oxacillin resistance. Confirmation of a *S. aureus* isolate as MRSA involves identification of the *mecA* through PCR as well as phenotypic characterization of oxacillin/cloxacillin. Phenotypic discrepancies may arise due to differences in growth condition (pH, osmolarity of media etc.), (18, 55) and variations in culturing techniques.

To date, farm level prevalence of MRSA on dairy farms in the U.S. has not been studied. As such, the major objective of this study was to establish the farm-level

prevalence of *S. aureus* and in particular, MRSA on dairies in Minnesota (MN). A second objective was to try to identify factors associated with the presence of MRSA on MN dairy farms.

Chapter 3: Isolation and characterization of *Staphylococcus aureus* including methicillin resistant strains from bulk tank milk of Minnesota dairies.

Introduction

The emergence of antibiotic resistant microorganisms on farm animal environments is a major public health concern. *Staphylococcus aureus* is an important opportunistic pathogen both in humans and in dairy cattle. It is also a common cause of mastitis in dairy cows (94, 101) - a primary reason for antibiotic use on farms. The use of antimicrobial agents on dairy farms as well as other food animal farms warrants concern due to the possibilities of development of resistant bacteria that may cause disease in humans (73). Although different antibiotic classes of drugs are used in animal health management and in human medicine, the selection of resistance to one drug class may lead to cross-resistance (73). Antibiotics on dairy operations are not only used to treat infections such as clinical mastitis but also used as preventive measures during dry cow therapy. Monitoring the emergence of drug-resistant pathogens in animal reservoirs is important, particularly in those with zoonotic potential.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a significant cause of healthcare associated (HA) and community associated (CA) infections (52). In addition, livestock associated (LA) MRSA, genotypically classified under clonal complex (CC) 398, has been detected among pigs and swine farmers in the Netherlands and other countries (25, 27, 50) and is known to cause infections in humans and animals (26, 57, 105). MRSA isolates are frequently multidrug resistant (MDR), which can result in higher costs, longer length of treatment, and higher rates of hospitalization and

comorbidities (20, 67). MRSA was first reported from bovine sources in 1975 (29), although occasionally since then (34, 42, 48, 96). The presence of MRSA in bovine milk and dairy environments poses potential risk to farm workers, veterinarians (48, 56), and likely risk to in-contact farm animals.

There is a need for studies to address the prevalence of drug-resistant *S. aureus*, particularly MRSA, in US dairy environments. Few studies have assessed the presence of MRSA in dairy herds in the US. MRSA prevalence in milk samples submitted to veterinary diagnostic laboratories in Michigan, Wisconsin, North Carolina, Virginia and other states in the US has been reported to range between 0-1.8% (8, 33, 59).

In comparison, prevalence rates of 29 to 35% have been reported in milk samples from Vermont, Pennsylvania and Louisiana respectively for pansusceptible or methicillin susceptible *Staphylococcus aureus* (MSSA), (22, 46, 69). It is important to understand the ecology of MSSA and MRSA in dairy herds to monitor for the emergence clonal types similar to LA MRSA- CC 398 (50).

S. aureus is also the third most reported cause of food borne diseases in the world (66, 109). Growth of *S. aureus* in foods leads to the production of staphylococcal enterotoxins (SEs), a cause of food poisoning (7). Contaminated milk and milk products have been frequently implicated in staphylococcal food poisoning (24). SEs are heat resistant and hence may be present even when *S. aureus* is not viable (72).

The aim of the present study was to characterize and document the prevalence and distribution of MSSA and MRSA in bulk tank milk samples. A secondary objective was to describe the nature of enterotoxin production by isolates.

Materials and Methods

Sampling Frame:

Fifty dairy farms in Minnesota (MN) were sampled in the study (Figure 1). Of approximately 4700 dairy farms in MN, about 2500 farms test regularly with MN Dairy Herd Improvement Association (DHIA). We selected a subset of herds from those registered with DHIA, based on their location in three major dairy regions in MN: Southeast region, South central region and the North central region. Herds were also categorized as small (40-99 head), medium (100-299 head) or large (≥ 300 head) according to the number of milking cows. Fifty herds were randomly selected from this pool, to equally represent the three dairy regions as well as the three herd size categories, and then solicited to participate in the study (participation was voluntary).

A questionnaire documenting farm management practices was completed by the 50 farms at the beginning of the study (early spring, 2009), and again at the end of the study (late fall, 2009). Bulk tank milk (BTM) samples were collected, in duplicate, on three consecutive days for each season: spring, summer and fall in 2009. Samples were frozen on the farm (-20ºC), then shipped on ice to the University of Minnesota, Laboratory of Udder Health. Samples collected from three consecutive days were pooled for a single herd from a single season giving a total of 150-pooled BTM samples (50 each from spring, summer and fall) for *S. aureus* assessment.

Isolation of MSSA:

Previously frozen bulk tank samples were thawed at room temperature. *S. aureus* was cultured from pooled milk by direct plating of milk onto Factor media (University of

Minnesota, St. Paul) using a spreader, and incubated at 37ºC for 24-48 h. *S. aureus* colonies were identified on the basis of colony morphology and confirmed using the Sure-Vue® Color Staph ID confirmatory test (Fisher Healthcare, TX). Isolates were transferred to Lauria-Bertani (LB) broth (BD Difco, Becton Dickinson Co., MD) and DNA was extracted using the QIAamp DNA mini kit (Qiagen Sciences, Maryland, USA) per manufacturer's instructions. All isolates were also tested for growth on MRSA *Select*™ plates (Bio-Rad Laboratories, WA) to determine methicillin susceptibility status of isolates.

Genotypic and phenotypic characterization of MSSA:

All MSSA isolates were confirmed as *S. aureus* by species specific *16s* rDNA PCR using the primers: 16s1 (5'CAG CTC GTG TCG TGA GAT GT 3'), 16s2 (5'AAT CAT TTG TCC CAC CTT CG 3') (17). *16s* rDNA amplicons were sequenced using regular Sanger sequencing at the Biomedical Genomics Center (BMGC), University of Minnesota using a primer concentration of 3.2 pmole and approximately 20 ng of amplified DNA as template (http://www.bmgc.umn.edu/facilities/sequencing/services/sequencing/home.html). Sequences obtained were compared to a reference *S. aureus 16s* rDNA sequence (Accession AM980864) using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

Staphylococcal protein A or *spa* typing was carried out for all *S. aureus* isolates as described (85). *Spa* types were obtained using the sequence comparisons against egenomics (http://tool.egenomics.com) and ridom (http://www.spaserver.ridom.de)

databases.

MSSA isolates were screened for their antimicrobial agent susceptibility patterns using the Kirby Bauer disk diffusion method using commercially available antibiotic disks (BBL, Sparks, MD). Interpretations of susceptible or resistant phenotype assignments were performed according to the Clinical and Laboratory Standards Institute guidelines.

Isolation of MRSA:

MRSA detection involved a two-step enrichment procedure with the inoculation of pooled milk into Mueller Hinton broth (Teknova Inc., CA) supplemented with 6.5% NaCl (24 h at 37ºC) followed by phenol red mannitol broth supplemented with 4mg/L oxacillin (24 h at 37ºC). The samples were then plated on MRSA *Select*™ media (Bio-Rad Laboratories, Hercules, CA), a selective media for detection of MRSA, for 24 h at 37ºC. If growth was detected, colonies were transferred to CNA plates (BBL, Becton Dickinson Co., MD, U.S.A) for 24 h at 37ºC to check for β-hemolysis. Isolates were further confirmed as *S. aureus* by the coagulase test using coagulase plasma, rabbit with EDTA (BBL, Becton Dickinson Co., MD). Thus selectively enriched isolates that grew on MRSA Select plates and showed classic positive β-hemolysis in CNA media as well as coagulase positive were considered as suspect MRSA. These isolates were then transferred to LB broth and DNA was extracted using the QIAamp DNA mini kit as previously mentioned.

Genotypic and phenotypic characterization of MRSA:

All suspect MRSA isolates were confirmed as *S. aureus* by *16s* rDNA sequencing as previously mentioned for MSSA. Detection of the *mecA* gene encoding the modified

penicillin binding protein PBP 2a was performed on confirmed *S. aureus* isolates. A *mecA* PCR was performed using a primer set targeting an upstream region of *mecA* - mecA1 (5'GTA GAA ATG ACT GAA CGT CCG ATA A 3') and mecA2 (5'CCA ATT CCA CAT TG TTC GGT CTA A 3'). *mecA* sequences were obtained by sequencing as described for *16s* rDNA and compared for sequence similarity to a reference gene (Accession No. EU790490) using BLAST (ncbi.nlm.gov). The *mecA* PCR was repeated for confirmation at the Minnesota Department of Health (MDH) by real-time PCR using a second set of established primers described by Killgore et al., (51). The *mecA* PCR performed using the primers from Killgore et al., was also repeated in our laboratory for all suspect MRSA isolates. The use of two different primer sets (Killgore et al., primer set and primer set designed by our lab), which target the upstream and downstream regions of the *mecA* gene, define the mediation of methicillin resistance by this gene. Further, SCC*mec* typing for the suspected MRSA isolates was carried out as outlined by Zhang at al. (108) to determine *mec* gene classes and *ccr* (cassette chromosome recombinase) types present in these isolates and assign SCC*mec* types respectively.

Genotypic characterization involved *spa* typing for all suspect MRSA isolates. All suspect MRSA isolates were further characterized by multilocus sequence typing (MLST) as described by Enright et al.(32). Sequence types were assigned using an established MLST database (http://www.mlst.net). These isolates were also characterized using pulsed-field gel electrophoresis (PFGE) using standard CDC protocols at MDH (61).

All suspect MRSA isolates were subjected to two different Sensititre antimicrobial

agent panels: one at the University of Minnesota, Veterinary Diagnostic Laboratory (VDL) and the other at the Minnesota Department of Health (MDH). Sensititre panels are commercially available microdilution trays which provide MICs against antibiotics tested. All antimicrobial agent panels included oxacillin to be applied as an indicator of methicillin resistance. All interpretations of susceptible, intermediate and resistant were done according to CLSI M100-S20 standards.

PCR amplification of genes encoding staphylococcal enterotoxins and PVL in suspect MRSA isolates:

The genomic DNA was extracted from the isolates using the QIAamp DNA mini kit as previously mentioned and used to screen for the following enterotoxin genes by PCR: *sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, seu* and *tsst* in addition to the Panton Valentine Leukocidin (PVL) toxin gene. PCR for the 19 toxin genes (97) and PVL (58) were performed using previously published methods.

Detection of staphylococcal A, B, C, D, E production in suspect MRSA isolates:

The suspected MRSA isolates were grown in LB media overnight at 37 ºC. The supernatant of the cultures were collected by centrifugation at 3500g for 5 min at 15 ºC. Sterile filtration of the supernatant was carried out using Millipore steriflip membranes (0.22µm) to avoid transfer of microorganisms to the assay. The commercially available RIDASCREEN SET A, B, C, D, E (R-Biopharm GmbH, Germany) sandwich immunoassay was used for the detection of staphylococcal enterotoxins (SE) in the supernatants of culture fluids. Protocol was followed as per manufacturer instructions for the enzyme immunoassay. Optical density at 450 nm (OD450) results were interpreted

using cut off values derived by adding 0.15 to the average negative control OD450. Samples with OD450 less than the cut-off value were considered negative while those with equal to or greater than the cut-off were considered positive for the respective toxin.

Results

MSSA- Prevalence and Characterization:

Of the 93 *S. aureus* isolates recovered from bulk tank milk (BTM) analysis, none showed growth on selective media for MRSA, the MRSA *Select*™ media (Bio-Rad Laboratories, Redmond, WA), indicating they were all methicillin susceptible. All isolates were confirmed as *S. aureus* by a 98-100% sequence similarity of 1*6s* rDNA to the reference gene (Accession AM980864). Ninety-three MSSA isolates were detected from the 150 bulk tank milk (BTM) samples - a 62% prevalence at the bulk tank level Herd level prevalence of MSSA was 92% (42 of 50 farms, *S. aureus* detection in at least one season in 2009 was considered as positive). Of the 93 MSSA isolates, 54 were pansusceptible by disk diffusion method, 15 were resistant to at least one antibiotic while the remaining 24 were resistant to 2 or more antibiotics (Figure 2). Fifteen isolates were resistant to 2 or more classes of antibiotics and thus classified as multidrug resistant (MDR). Of the 93 isolates, 29% were resistant to cloxacillin, 17% to ampicillin, 16% to penicillin and tetracycline each, 7% to erythromycin, 5% to pirlimycin, 4% to novobiocin, 3% to streptomycin and 1% were resistant to ceftiofur and cephalothin each as determined by Kirby Bauer disk diffusion method.

MSSA isolates were classified into the following spa types: t529 (*n*=25), t034 (*n*=10), t359 (*n*=2), t189 (*n*=2), t203 (*n*=2), t044 (*n*=1), t084 (*n*=1), t1166 (*n*=1), t267

(*n*=4), t2734 (*n*=1), t337 (*n*=1), t4173 (*n*=1), t521 (*n*=1) and unknown or new spa types (*n*=41). Amongst the 15 MDR isolates, t034 (*n*=5) and t529 (*n*=4) spa types dominated. MSSA isolates showed most resistance to beta-lactam antibiotics followed by tetracyclines across all 3 seasons (Figure 3).

MRSA- Prevalence and Characterization:

Eight isolates were identified as suspect MRSA from a duplicate set of BTM samples using selective enrichment as mentioned previously. These isolates amplified an upstream region of *mecA* and their sequences aligned (98-100% nucleotide identities) with *S. aureus mecA* gene sequences in the NCBI databases. Species specificity of the suspect MRSA isolates was also confirmed by a 98-100% sequence similarity of 16*s* rDNA to the reference gene (Accession AM980864). However, the results of a TaqMan-PCR assay from Minnesota Department of Health (MDH) using an established primer set showed that only 2 of the 8 suspect MRSA isolates contained the *mecA* gene segment. Repetition of the MDH m*ecA* gene PCR in our lab using conventional PCR produced results consistent with those of MDH. Sequence alignment of primers used in our lab and those of Killgore et al., (51) showed that the primers amplified different regions of the ORF of the *mecA* gene (regions separated by 500 bp). Furthermore, the Sensititre results of the 8 suspected MRSA isolates verified that the two *mecA* positive isolates were MRSA because they were resistant to oxacillin with MICs > 32 µg/ml. Another isolate (Fa25) showed low-level resistance to oxacillin on only one of the panels but did not carry the *mecA* gene in the MDH assay and hence was not considered as MRSA. The remaining 5 isolates were all sensitive to oxacillin confirming them as false positives.

Hence, of the 8 suspect MRSA isolates, only 2 were confirmed as MRSA, while the rest six were false positives.

Two isolates (1.3%) were confirmed as MRSA from 150 BTM samples after enrichment and screening for the presence of the *mecA* gene. The prevalence of MRSA in BTM was 1.33% (2 of 150) while the herd prevalence of MRSA was 4% (2 of 50).

The two MRSA isolates each showed resistance to oxacillin at MICs > 32 µg/µl. The first MRSA isolate (Sp 19), was resistant to to β -lactams, lincosamides, macrolides, 1st generation, 2nd generation and 3rd generation cephalosporins. The second MRSA isolate (Sp 12) was resistant to floroquinolones, carboxypenicillins and oxalzolidones in addition to the antibiotic classes that Sp19 was identified as being resistant. Two of the six false positives (suspect MRSA isolates) were MDR while the rest four were pansusceptible to all antibiotics tested. The two MDR MSSA isolates were resistant to β -lactams, lincosamides, macrolides, 1st generation, 2nd generation and 3rd generation cephalosporins, carboxypenicillins and tetracyclines.

Subtyping using MLST, PFGE, *SCCmec* typing and *spa* typing was performed for the 2 MRSAs and 6 suspect isolates (Figure 4). One MRSA isolate (Sp12) carried a composite profile of (unknown spa type-PFGE type:close to USA 100 -ST 5- SCC*mec* type II). The second isolate (Sp19) shows a composite profile of (*spa* type:t121-PFGE type:USA 300-ST 8- SCC*mec* type IVa). Other PFGE types in the suspect MRSA include: USA 400 (*n*=2), closest to USA 400 (*n*=2) and non-typable (*n*=2). MLST types found in the false positives include: ST 352 (n=4), ST 398 (n=1) and unassigned ST. No SCC*mec* -types could be obtained for any of the false positive isolates. Spa type t121 was

the only spa type identified among the 2 MRSA isolates while types t034 (n=2), t359 (n=2), t2734 (n=1) and unknown type (n=1) were detected in suspect MRSA isolates.

Presence of SE genes and SE production in suspect MRSA isolates:

The 2 MRSA and 6 isolates that were first misclassified as methicillin resistant were further analyzed for toxin production profiles and presence of enterotoxin genes. Although only 2 of the 8 isolates were confirmed as MRSA by detection of *mecA* and phenotypic expression of methicillin resistance, toxin data from the 6 suspect isolates was presented to depict the toxigenic potential of non-methicillin resistant strains isolated from milk. MRSA isolate Sp12 produced SEB, SEC and SED while isolate Sp19 produced SEC, SED and SEE (Table 1). Among the 8 *S. aureus* isolates analyzed, the order of SE production (produced by most number of isolates) is as follows: SEC> SED> SEB, SEC. Enterotoxin genes were detected from most isolates (Table 1) and showed following frequency distribution: SER>SED>SEI, SEJ> SEB, SEC, SEE, SEU, SEM, SEO, SEQ. Panton-Valentine Leukocidin (PVL) gene was detected in one isolate alone: the *Sp 19* MRSA isolate.

Discussion:

MRSA has increasingly been recognized in farm animal populations in recent years. Some studies have investigated MRSA prevalence in pig populations in North America (50, 89); however, very few studies have analyzed and characterized the presence of MRSA in U.S. dairy cattle or in U.S. bulk tank milk.

The 50 farms sampled in our study are representative of 2.3% of all dairy herds enrolled in the MN DHIA (50 of 2159 farms). The mean milk production in terms of rolling herd averages (lbs. of milk/cow/year) for the 50 farms (21,586 lbs.) were reflective of the mean rolling herd averages of the farms enrolled in MN DHIA (21,329 lbs.). The average number of cattle in our 50 farms (580 cows/herd) was higher compared to that of the farms enrolled in the MN DHIA testing cohort (121 cows/herd) in 2009, but this is because study farms were intentionally selected for solicitation to represent equally both small, medium and large herd sizes.

Our results suggest that there is a low prevalence of MRSA in MN dairy herds. This is inconsistent with the NAHMS Dairy 2007 study (included samples from MN) which found no MRSA in bulk tank milk samples (99). It may be that the current study was able to detect MRSA, while the national NAHMS study did not, because the laboratory methodology used in the current study were more robust and used selective enrichment for oxacillin resistant *S. aureus*. By comparison, the NAHMS study used two parallel isolation methods: one which involved direct plating of BTM onto a selective media, the CHROMagar MRSA plates and another which involved plating on trypticase soy agar with 5% sheep blood and 0.1 esculin to obtain staphylococcal colonies which may have resulted in

reduced sensitivity for detection of MRSA. Consistent with our findings, other countries have reported varying prevalence - 1.4% (Switzerland) (42), 1.5% (Japan) (41), 2.4% (Korea) (63), 0-7.4% (Belgium) (96), 5.1-16.7% (Southwest Germany) (91) of MRSA in milk from individual cows. Similarly, in the United States, previous studies have reported zero to low occurrence of MRSA among *S. aureus* isolates from milk samples selected from individual cows – 0.6% of 846 *S. aureus* isolates (Michigan) (33), 1.8% of 2132 *S. aureus* isolates (Wisconsin) (59) and 0% of 357 *S. aureus* isolates (North Carolina and Virginia) (8). Recently, a study of *S. aureus* from mastitic cattle in India reported a high cow-level prevalence of MRSA-13% of *S. aureus* isolates (14 of 107 samples) (54). Such high prevalence has not been reported in studies from U.S or Europe, with the exception of Southwest Germany (91). However, for the India study, only 10 of 14 isolates classified as MRSA by the authors showed the presence of *mecA* gene by PCR.

Although eight isolates were detected on MRSA *Select*™ plates, six of these did not amplify the *mecA* by MDH primers nor showed high MICs to oxacillin. A segment of the *mecA* was amplified suggesting a possible truncation in the ORF region. It is likely that these isolates showed poor expression of *mecA* genes or over production of beta-lactamase as they remained susceptible to oxacillin +2% NaCl but resistant to penicillin (43, 63). Another factor that may explain this discrepancy could be that phenotypic expression of resistance varies with growth condition (pH, osmolarity of media etc.) (18, 55). On the other hand, higher MICs of oxacillin resistance (of the order of 16ug/ml) have been observed in MRSA isolates upon induction (i.e culturing in the presence of oxacillin) which previously exhibited low MICs of 1-2 ug/ml (86). Hence, false positives

could also arise due to limitations in the microbiological detection methods. Because of this, all suspect MRSA isolations from animal environments should be subjected to Sensititre based MIC detection and confirmation by *mecA* PCR (51).

In a recent study of MRSA isolated from mastitic milk samples collected from individual cows, 100% were resistant to erythromycin, clindamycin, chloramphenicol and gentamicin (95). In another study in Switzerland, two MRSA isolates from 142 mastitic milk samples were resistant to ampicillin, ceftiofur, clindamycin, erythromycin, oxacillin, penicillin and tetracycline (42) while Vanderhaghen et al. (96) reported resistance to tetracycline with frequent resistance to macrolides, lincosamides and aminoglycosides in MRSA from mastitic milk. Resistance profiles of MRSA from the previous two studies closely resemble the profiles of both MSSA and MRSA in our current study. Further studies are needed to analyze the relationship between patterns of antibiotic use on farms and the emergence of resistance to specific drugs.

The most prevalent *spa* type among MRSA isolated from bovine sources is t011 as is corroborated by reports from Germany, Belgium and Switzerland (34, 42, 91, 96). MRSA from bovine mastitis samples in Germany and Belgium (34, 96) also included other spa types - t034, t2576 and t567. Limited data on *spa* type distribution are available for MSSA isolates from bovine sources. Of the spa types obtained in our study, two of the MSSA isolates belonged to the type found in Germany (34), t034, while one MRSA isolate belonged to type t121 which is common throughout France, Belgium, Germany, Switzerland and U.S.A and associated with MLST: ST 8 (http://www.spaserver.ridom.de).

Isolate *Sp12* carried a composite genotype of ST5- close to USA 100- SCC*mec* type II which is a commonly reported genotype among hospital-associated MRSA while isolate *Sp 19* carried a composite profile of ST8- USA 300- SCC*mec* type IVa reported in community-associated MRSA lineages with the smaller SCC*mec* cassette IV (61). In contrast, MRSA isolates from mastitic milk in Switzerland and Belgium belonged to the MLST type ST 398 which is a LA MRSA associated sequence type (34, 42, 61). Bovine MRSA isolates from Germany were also untypable by *Sma* I PFGE (34, 42). Such untypable - ST 398 isolates have been frequently detected from MRSA isolates from pigs in the U.S as well as in other countries (25, 89). Two of the suspect MRSA isolates which were later confirmed susceptible to methicillin show spa type, MLST and PFGE composite profiles similar to those seen in LA MRSA genotypes indicating such genotypes are present in MN dairy farms but remain susceptible to methicillin. These two isolates were also MDR and showed resistance to tetracyclines as is commonly observed in LA MRSA isolates.

Although the prevalence of MRSA is low in MN dairies and *S. aureus* is rendered inviable by pasteurization, it may pose potential hazards in situations where raw milk is utilized directly for consumption. At present, raw milk sales are legal in MN if the consumer purchases directly from the farm. Also, many dairy producers and their families consume raw milk produced on the farm. Two surveys conducted in 1999 and 2001 by Jayarao et al., (44, 45) reported that 60% of dairy producers in eastern South Dakota and Western Minnesota consumed raw milk. In the current study, 36% of the 50 participant farms reported consumption of unpasteurized raw milk produced on the farm.

Production of heat stable toxins capable of retaining immunological and biological activity following pasteurization is also a cause for concern (72). Of the 8 isolates analyzed for toxin production (2 MRSA, 6 MSSA), 7 showed production of at least one enterotoxin which could be explained based on the relatively high percentage of classical SE producing strains from bovine subclinical mastitis (9, 14, 23, 79). As previously observed in literature, none of the *S. aureus* isolates from milk produced SEE (14, 64, 79, 111) but we found one SEE producing MSSA isolate in this study. Only isolates positive for *sed* were also positive for *sej*. This confirms work by Zhang et al. (109) which shows that both genes were likely present on the same plasmid. Albeit very low, these findings pose the potential food borne risk of *S. aureus* and possibly MRSA (47, 53).

In summary, MDR MRSA capable of producing multiple staphylococcal enterotoxins and carrying genotypes commonly isolated in both healthcare and community settings were found in two (of 50) MN dairy herds. Different states of interaction can exist between *S. aureus* and its host: infection, carriage, colonization and contamination (3). Healthy humans can act as carriers if persistently or intermittently colonized by MRSA (colonization is a major risk factor) and can play a role in its spread between animals, the dairy environment and the community. Transmission of MRSA between humans and animals, in particular between cows and humans, has been reported (48), but based on our findings we expect this risk to be low as there is a low farm level prevalence of MRSA. The prevalence of MRSA in bulk tank milk is however a potential public health risk which may spread between animals. *Staphylococcus aureus* transmission is dynamic and involves human, animals and likely the farm production

environment. Further monitoring of the resistance status of *S. aureus* in dairy environments is important and more studies are needed to help identify critical areas that allow for milk contamination and spread.

Table III-1: Staphylococcal enterotoxins produced and toxin genes detected for MRSA and MSSA isolates.

*Only Sp12 and Sp 19 are MRSA isolates, rest were suspect MRSA but confirmed as MSSA isolates.

** SE denotes Staphylococcal enterotoxin. SEB, SEC etc refer to staphylococcal enterotoxin B, staphylococcal enterotoxin C and so on.

*** PVL refers to Panton Valentine Leuocidin toxin.

Staphylococcal enterotoxins produced and toxin genes detected for MRSA and MSSA isolates.

Sample Id	Organism	Toxin production analysed for SE					Toxin genes detected by PCR
		A	B	C	D	E	
Sp 12	*MRSA*	-	+	+	+	-	SED, SEG, SEI, SEJ, SEM, SEO, SER
Sp 19	*MRSA*	-	-	+	+	+	SED, SEQ, PVL
Fa 6	MSSA	-	-	+	-	-	SER
Fa 20	MSSA	-	-	+	+	-	SED, SER
Fa 25	MSSA	-	-	+	-	-	SED, SEE, SEU, SER
Fa 42	MSSA	-	-	+	+	-	SER
Fa 43	MSSA	-	-	-	-	-	SER
Fa 48	MSSA	-	+	+	+	+	SEB, SEC, SED,SEI,SEJ,SER

Figure III-1: Spatial distribution of participating dairy herds in Minnesota (*n*=50).

The fifty study farms are represented as white crosses on the physical map of Minnesota. These farms were selected to equally represent the three major dairy regions: South central, southeast and north central regions in Minnesota as well as to have an equal distribution of small (40-99 cattle), medium (100-299) and large (>300) farms.

Spatial distribution of participating dairy herds in Minnesota (*n*=50).

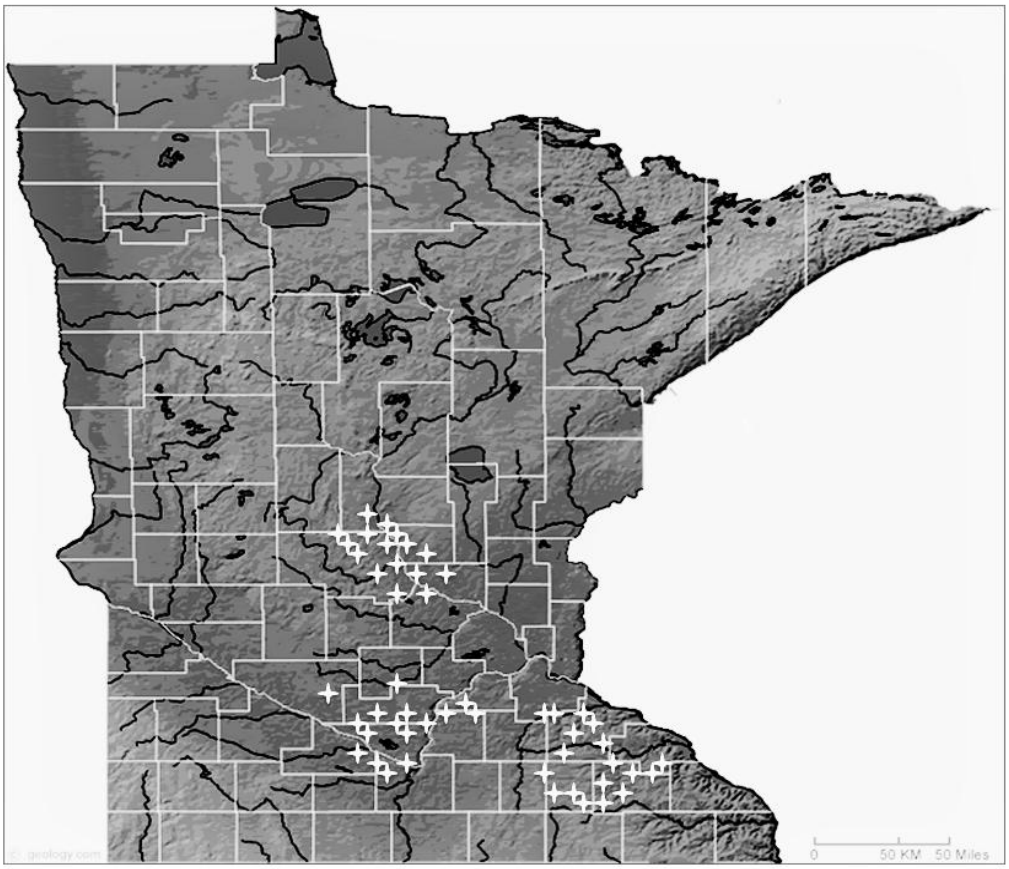


Figure III-2: Classification of MSSA isolates according to antibiotic resistance against different antibiotics tested using Kirby Bauer method.

Of the 93 MSSA isolates, 54 were pansusceptible to all antibiotics tested by Kirby-Bauer method, 15 were resistant to at least one antibiotic while remaining 24 were resistant to 2 or more antibiotics.

Classification of MSSA isolates according to antibiotic resistance against different antibiotics tested using Kirby Bauer method.

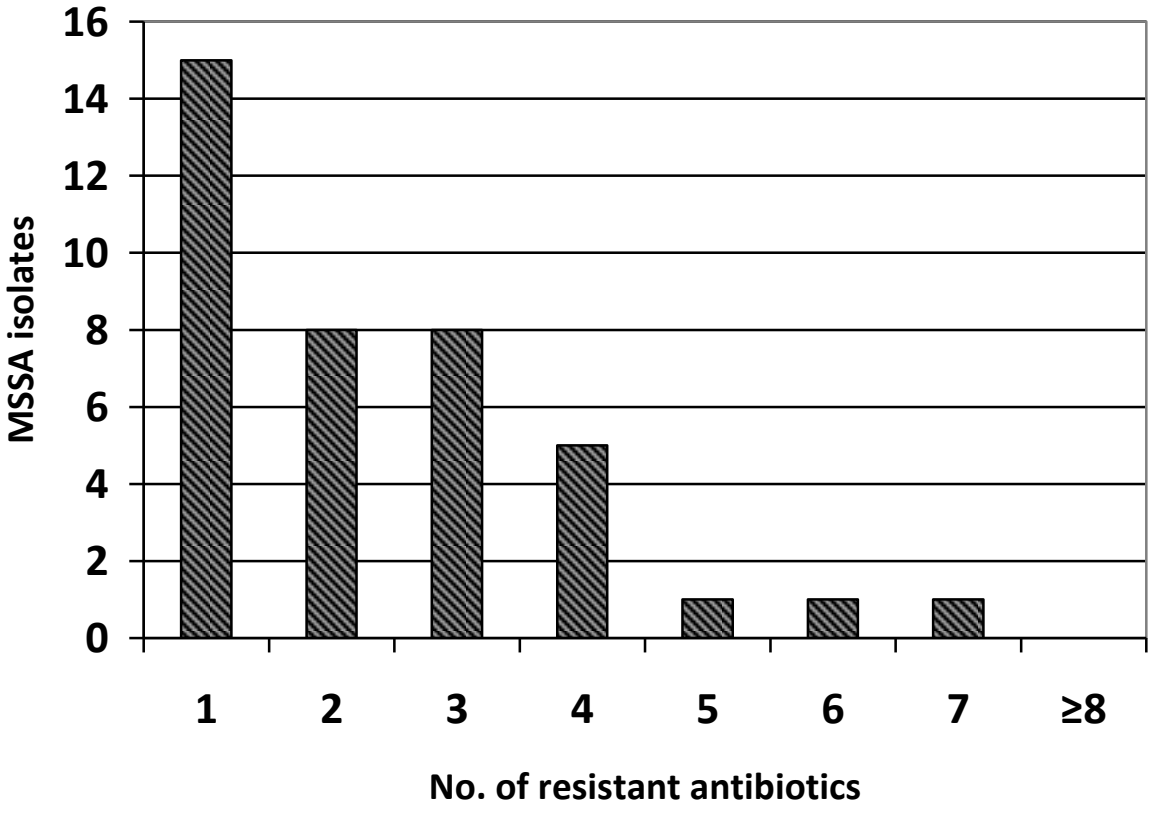


Figure III-3: Distribution of antimicrobial resistance to different classes of antibiotics among the 93 MSSA isolates across the 3 seasons in 2009.

Antimicrobial agent resistance was determined by Kirby Bauer method for all MSSA isolates with individual antibiotic disks. Proportion of MSSA isolates varied among the three seasons: Summer (*n*=16), spring (*n*=41) and fall (*n*= 36).

Distribution of antimicrobial resistance to different classes of antibiotics among the 93 MSSA isolates across the 3 seasons in 2009.

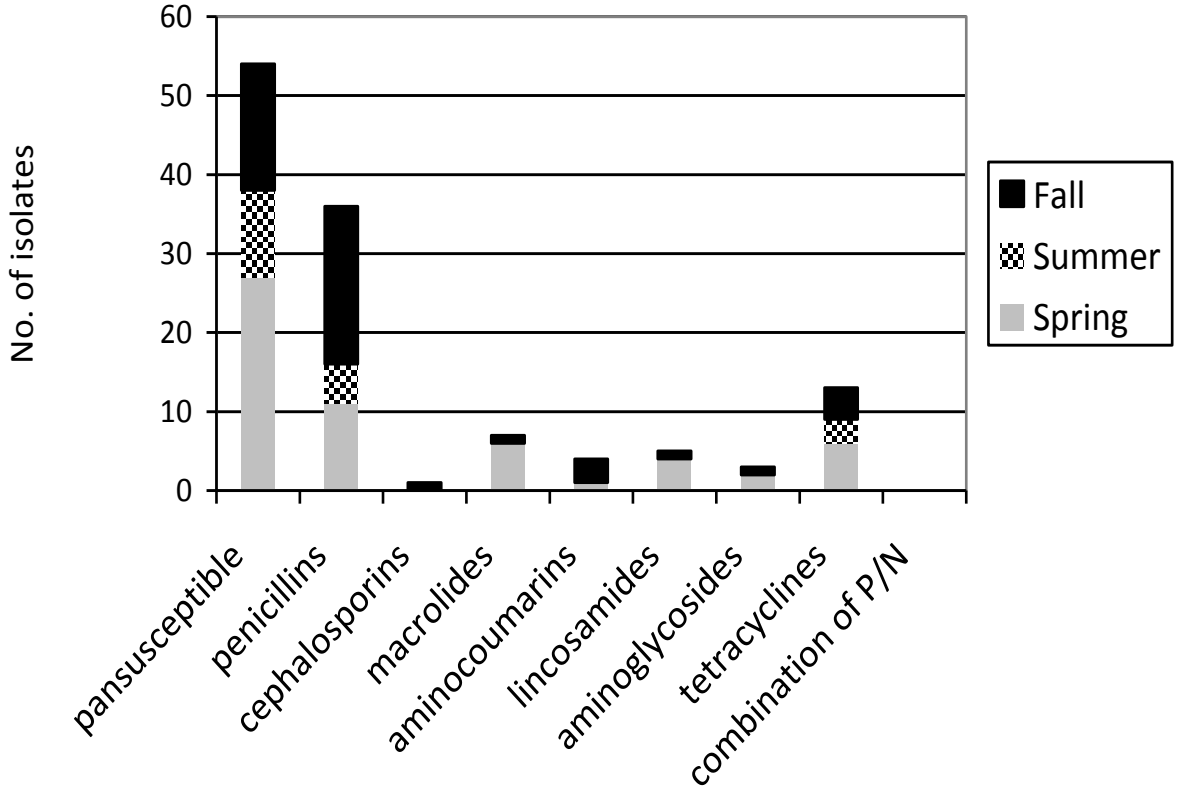
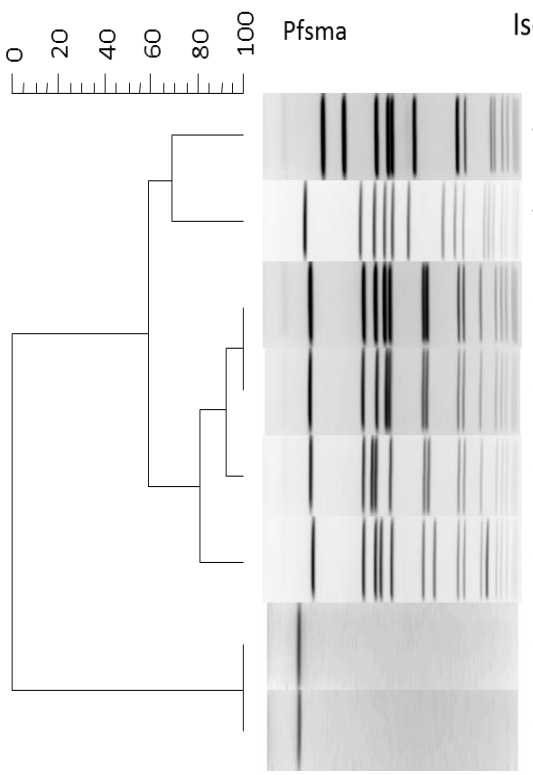


Figure III-4: PFGE patterns of MRSA and staphylococcal enterotoxin producing MSSA compared with subtypes obtained by MLST, *spa* typing and SCC*mec* typing.

The composite genotype obtained by the combination of PFGE pulsotype with MLST sequence type, *spa* type and SCC*mec* type provide important information about the epidemiology of the MRSA and MSSA isolates.

PFGE patterns of MRSA and staphylococcal enterotoxin producing MSSA compared with subtypes obtained by MLST, spa typing and SCCmec typing.



Isolate	PFGE	MLST	*Spa* Type	Mec Type
SP12	CLOSEST TO USA 100	ST 5	Unknown type	II
SP19	USA 300	ST 8	t 121	IVa
FA6	USA 400	ST 352	t 359	None
FA42	USA 400	ST 352	t 2734	None
FA20	CLOSEST TO USA 400	ST 352	t 359	None
FA48	CLOSEST TO USA 400	NEW ST	Unknown type	None
FA43	Non Typable	ST 398	t034	None
FA25	Non Typable	ST 352	t034	None

Chapter 4: Risk factor Analysis for the presence of multidrug- and methicillin-resistant *Staphylococcus aureus* in Minnesota dairy farms

1. Introduction

Mastitis is a costly infectious disease of dairy cattle worldwide. Antibiotics are commonly used to treat clinical mastitis and hence mastitis is a primary cause of antimicrobial use on dairy farms. *S. aureus* is a significant cause of bovine mastitis as well as an important opportunistic pathogen in humans and other animals (101). It has a tendency to acquire antimicrobial resistance rapidly. This has been witnessed by the emergence of penicillin resistant *S. aureus* following the introduction of penicillin in 1961 as well as the emergence of methicillin resistant *S. aureus* (MRSA) following the introduction of the synthetic beta-lactam antibiotic: methicillin. MRSA often exhibit resistance to all β-lactam antibiotics and frequently to multiple other classes of antibiotics such as tetracyclines, lincosamides etc. (49). As a result, infections caused by MRSA are difficult to treat due to the limited choices of effective antibiotics available as well as enhanced virulence displayed by some MRSA compared to MSSA in some cases.

Historically, three distinct lineages of MRSA have emerged: in hospitals, associated with large scale antibiotic use and known as hospital associated MRSA (HA MRSA), in communities associated with certain risk factors such as crowded unhygienic conditions, among people with compromised skin integrity and those which share contaminated objects-known as community associated MRSA (CA MRSA), and finally in farmed animals such as pigs, cattle, poultry and other animals- known as livestock associated or LA MRSA (102).

There is an ongoing debate about the use of antibiotics in animal husbandry, for therapeutic and sub-therapeutic purposes and the emergence and dissemination of multiple antibiotic resistant zoonotic bacterial pathogens. The use of antibiotics in animal farming is controversial because of the possibility that such use could select for resistant strains of pathogens that could be transferred to humans via contact with animals or animal products, including the ingestion of contaminated food and water (73).

The use of antibiotics for dry cow therapy (DCT) for both preventive and therapeutic purposes in dairy heifers has been well documented (37). The different classes of antibiotics available for the intramammary (IMM) treatment of mastitis in dairy cows includes β- lactams (penicillin,cephapirin, ceftiofur, amoxicillin, betacillin and cloxacillin), macrolides (erythromycin), coumarines (novobiocin), and lincosamides (pirlimycin) (4). Antibiotics are also used to treat other diseases or infections on farm such as: respiratory and uterine diseases and infectious foot disease. Compounds used to treat foot infections include sulphonamides, β-lactams, tetracylines and lincomycin while compounds used for treatment of respiratory disease or metritis include ceftiofur and other β-lactams, tylosin, tilmicosin, florfenicol, tetracyclines and sulphadimethoxine (1).

Data from a national survey of dairy herds (USDA/APHIS/VS/CEAH, 2005) suggested that greater than 75% of dairy farms used IMM DCT in all cows. It also reported that cephapirin (42%) was the most commonly used drug followed by penicillin/dihydrostreptomycin (32%) and cloxacillin (13%) (76).

With widespread use of antibiotics for preventive and therapeutic purposes on dairy farms, few studies have statistically analyzed the relationship between antibiotic use and

emergence of antibiotic resistant pathogens on dairy farms. Poll and Ruegg (75) reported a heterogeneous relationship of *S. aureus* MICs with drug usage on farm and concluded that a clear dose response interaction was not observed. Further, they also observed no significant associations between level of antibiotic exposure (in organic vs. conventional farms) and MICs of mastitis pathogens (75). Erskine et al. also detected no indication of increased resistance of mastitis isolates (including *S. aureus*) to commonly used antibiotics, and instead reported an increase in proportion of isolates susceptible to ampicillin, penicillin and erythromycin over a seven year period (33). Rajala Schultz et al analyzed the susceptibility patterns of mastitis pathogens isolated at calving from first lactation and older cows and reported no significant difference in the proportion of resistant isolates between first lactation and older cows (80). The study conducted by Roesch et al in Switzerland compared the occurrence of resistant mastitis pathogens between organic and conventional dairy farms and reported no significant differences between the two (81).

Although some studies have analyzed the relationship between the MIC's of mastitis pathogens and emergence of antibiotic resistance, limited information is available about the influence of farm management practices, including antibiotic use patterns, on the emergence of resistant pathogens. Such data is important for surveillance purposes, as differences in management may have significantly affected the development of antibiotic resistance in mastitis causing *S. aureus*. The objective of this study was to investigate if there are specific farm management practices or factors that are associated with the the occurrence of multidrug resistant MSSA (MDR MSSA) as well as MRSA

from bulk tank milk (BTM) on Minnesota dairy farms.

2. Materials and Methods

2.1 Data Collection

2.1.a Farm sampling

Fifty farms in Minnesota were selected as described in Chapter 3 to participate in the study.

2.1.1 Milk Sampling

Bulk tank milk (BTM) samples were collected from Minnesota dairy herds (n = 50) over three seasons: spring, fall and summer in 2009. Details on study design and sample collection are described in Chapter 3. A total of 150, pooled, BTM samples (50 each from spring, summer and fall) were analyzed for *S. aureus* assessment.

2.1.2 On farm data collection

In order to collect data on risk factors, a comprehensive herd management questionnaire was administered to each of the fifty farms in spring 2009. The questionnaire was divided into the following categories: A. Producer information, B. Inventory- Herd size/expansion status, C. Production, D. Housing, E. Feed and Water-Adult cows, F. Bedding, G. Manure management, H. Calf management and feeding, I. Health management and antibiotic use. A shorter follow-up questionnaire was administered in Fall 2009 to record any changes in diet, antibiotic use practices, or inventory of the dairy cows. A copy of both questionnaires is appended at the end of this chapter.

2.2 Bacteriological analysis of BTM

Isolation of methicillin susceptible *Staphylococcus aureus* (MSSA) and MRSA was

carried out as outlined in Chapter 3. Antibiograms for MSSA and MRSA were obtained by the Kirby Bauer method and Sensititre method respectively as described in detail in Chapter 3. MSSA isolates which showed resistance to two or more classes of antibiotics were classified as MDR MSSA.

2.3 Statistical Analysis

2.3.1 Database management

Data from the questionnaires were coded and entered into a Microsoft (MS) Access database. From this database, risk factors relevant to the emergence of multiple drug resistant *S. aureus* were chosen and entered into a MS Excel database along with the laboratory data for MDR MSSA and MRSA. Occurrence of MRSA or MDR MSSA was coded as (0 or 1) binary variable with 1 representing presence, and 0 the absence, of the respective organism.

Commercial antibiotics used on farm for treatment of various diseases and in DCT were classified according to their active antibiotic compound and assigned antibiotic classes respectively (table 1). The antibiotic classes data was used to analyze relationship between antibiotic use pattern and occurrence of resistant pathogens.

2.3.2 Statistical methods

Summary statistics and frequency distributions for the variables of interest were calculated using SAS® 9.2 software. A total of 64 independent variables were analyzed for their association with each outcome of occurrence of MDR MSSA or MRSA on MN dairy farms (table 13). The variable herd size describes the total number of cows on farm including the number of milking cows. Univariate analysis, using the GLM procedure,

was carried out for each of the 70 individual variables and the following two outcome variables: i) the occurrence of MDR MSSA in the bulk tank sample as well as ii) the occurrence of MRSA in the bulk tank sample. The PROC GENMOD procedure from SAS® (version 9.2, SAS Institute, Cary, NC) was used to perform a GEE analysis of repeated measures on herds for the outcome of MDR MSSA or MRSA in those herds. This was done to account for the multiple bulk tank samples, which were taken on the same herds at 3 different points in time. For univariate analysis, a cut-off value of $P< 0.2$ was used for MDR MSSA models, while a cut-off value of $P<0.3$ was used for MRSA models, to screen for potential risk factors using unconditional associations. The potential risk factors which were identified from the univariate analyses were then carried forward and included in the multivariate model for occurrence of i) MDR MSSA and ii) MRSA. To build the final multivariate models, non-significant variables were sequentially removed using backwards stepwise elimination process. Any variables which showed values of $P<0.15$ but ≥ 0.05 in the final model were left in the model and reported as trends. Variables which showed $P<0.05$ in the final model were identified as significant associations.

In the final multivariate analyses, for each outcome of i) MRSA and ii) MDR MSSA, three different models were constructed to avoid overlap of herd management data and hence correlated data. The three models constructed were:

Model I: All Diseases Model: This considered variables describing antibiotic use for treatment or prevention of any disease or infection on farm;

Model II: Mastitis model: This considered variables describing only those antibiotics used in the treatment and prevention of clinical mastitis, including both intra-mammary and systemic drug use;

and

Model III: Other diseases model: This considered variables describing systemic antibiotic use for the treatment of any diseases other than mastitis (e.g. lameness, metritis and respiratory diseases on farm).

As a result, six models were constructed and analyzed for significant associations, three each for the outcomes MRSA and MDR MSSA.

3. Results

3.1 Descriptive Statistics

The 50 MN farms are representative of the 2159 MN Dairy farms enrolled with the DHIA and were selected as mentioned in Chapter 3. The summary statistics for the 50 dairy herds including the number of milk cows, total cattle, rolling herd averages (RHA) and output of milk per cow per day were generated (table 2). The average number of cattle in the 50 dairy herds was 580 with an average of 270 milking cows in each herd. Mean RHA was 21586 lbs of milk per cow per year with mean milk output of 74 lb/cow/day (table 2). Herds were chosen to be equally distributed among the three dairy regions (NW, SE and SW), herd size categories (small, medium, large) and herd status as open/closed (i.e herd status is open if animals are purchased from outside sources for farm operations and vice versa). All farms had Holstein breed cattle except for one Brown Swiss herd. All farms were conventional (i.e. not organic) (table 3). Highest percentage of farms (40%) recorded average bulk somatic cell counts of 200,000-299,000 cells/ml while 50% of farms recorded average bacterial counts of 0-4999 cell/ml (table 4). Eighteen of the 50 farms reported consumption of unpasteurized raw milk produced on farm. Four farms reported milk or meat antibiotic residue violations in the past one year proceeding the study period (table 4).

Practices of bedding management and sick cow housing were documented as they may play a role in spread of contagious pathogens. Seventy eight percent of farms reported no isolation of sick cows, organic bedding was commonly used among 62% of the farms (table 5). About 90% of farms reported 100% use of DCT during drying off

while the remaining 10% reported little (10-12%) or no use (0 %) of DCT (table 6).

Antibiotic use on farms was classified as IMM use in DCT, IMM use for treatment of clinical mastitis and systemic use for treatment of all diseases on farm. Based on this classification, 1st generation cephalosporins were most commonly used on farms for IMM DCT while 3rd generation cephalosporins were used for IMM treatment of clinical mastitis in lactating cows. Third generation cephalosporins were also most commonly used for systemic treatment of respiratory diseases, uterine infections or metritis and lameness (table 7). Third generation cephalosporins were also the most commonly used antibiotics overall among all diseases on 90% of farms (table 7).

Fifty-nine percent (55 of the 93) of MSSA isolates recovered from the 150 bulk tank milk samples were pansusceptible, 38 were resistant to at least 1 class of antibiotic while 16 were resistant to two or more classes and defined as MDR isolates (table 8). Most isolates were resistant to β-lactams followed by tetracycline class of antibiotics (table 9).

3.2 Univariate Analysis

The univariate associations (P<0.3) between management factors and the outcome of MRSA are shown in table 9, while the univariate associations (P<0.2) between management factors and the occurrence of MDR MSSA are shown in table 10. In the MRSA models, unconditional associations of independent variables which cleared the preliminary screening P-value of < 0.30 included:

- *Antibiotic residue violation in the past year,*
- *Routine use of DCT*

- Use of the following IMM antibiotics for DCT: β-lactams, β-lactam-aminocoumarin, 1st generation cephalosporin, 3rd generation cephalosporins.

- Use of following IMM antibiotics for treatment of mastitis: 1st generation cephalosporin, 3rd generation cephalosporins, organic/inorganic products and tetracyclines.

- Use of the following systemic antibiotics for treatment of other diseases: 1st generation cephalosporin, 3rd generation cephalosporins, β-lactams, tetracyclines, anti-inflammatory products and other organic/inorganic products.

- Overall use of the following antibiotics for treatment of any disease on farm: β-lactams, 1st generation cephalosporin, 3rd generation cephalosporins and anti-inflammatory products.

Independent variables which were identified as potential risk factors after univariate analysis for the MDR MSSA model ($P < 0.20$) included:

- *Farm region (NW, SW, SE)*
- *Herd size (indicates total cattle on farm including milk cows, dry cows, youngsock and bulls)*
- *Number of milk cows*
- *Residue violation in the past year*
- *Routine use of DCT*
- *Herd status as open/closed*
- *Use of following systemic antibiotics for treatment of diseases:*

3rd generation cephalosporins and tetracyclines for mastitis treatment, macrolides,

phenicols, teracyclines, anti-inflammatory and other organic/inorganic products for respiratory disease treatment, 3rd generation cephalosporins for lameness treatment, tetracycline, macrolides and phenicols for overall treatment of any disease on farm.

3.3 Final Multivariate Models for MDR MSSA:

Table 11 shows the risk factors associated with the presence of MDR MSSA in bulk tank milk, using an initial screening P value of <0.20. From this, the final three multivariate models were constructed as previously defined (Model 1 – Considered all drugs used for any disease; Model 2 – considered drugs used for mastitis therapy and prevention; Model 3 – considered drugs used for any disease other than mastitis):

The only explanatory variables that remained as significant in the final 3 models predicting the presence of MDR MSSA in bulk tank milk samples included: i) Region (decreased risk in herds from the NW and SE regions, as compared to the SW region), ii) Herd status (increased risk in 'closed' herds as compared to 'open' herds). The use of phenicol antibiotics in the herd in models 1 and 3, tended to be associated with a decrease in the risk for presence of MDR MSSA in bulk tank milk, but this was not statistically significant ($0.05 < P \leq 0.15$). Additionally, for Model 2 (considered antibiotics used only for mastitis therapy), the risk for presence of MDR MSSA in bulk tank milk tended to be reduced in herds that did not routinely use DCT, but this also was not significant ($P = 0.14$).

The odds of detecting MDR MSSA in the SW region were 1.3 times more than in NW region and 1.27 times more than SE region in all three models (statistically significant at $P<0.05$). Closed herds showed 1.1 times the odds (statistically significant at

95% confidence limits) for the presence of MDR MSSA compared to open herds in all three models (P<0.05). In models 1 and 3, the disuse of phenicols showed a greater odds of 1.09 times the use of phenicols on farm for the presence of MDR MSSA (P<0.05). Herds that routinely use DCT also showed an increased odds of 1.069 for the presence of MDR MSSA compared to herds that rarely used DCT (95% C.L: 0.8676,0.9708, P<0.05).

3.4 Multivariate models for MRSA

Multivariate modeling was carried out for the presence of MRSA in a bulk tank sample, similar to the approach previously described for the dependent variable: MDR MSSA. No significant associations were detected between any of the management factors included in the model after univariate analysis and the occurrence of MRSA.

4. Discussion and Conclusion

In concurrence with previous cow-level studies by Poll and Ruegg, 2007; Erskine at al.,2002; and Roesch et al. 2006 (33, 76, 81), the current study did not identify any statistically significant relationship between the use of various classes of antibiotics on the farm and the presence of MDR MSSA or MRSA in bulk tank milk samples. Although it is well established that long term use of antimicrobial agents in both human and veterinary medicine can lead to emergence of antimicrobial resistant bacteria (68), no studies have illustrated this with respect to MRSA in dairy environments.

According to Erskine et al. (33) the current evidence does not support the idea of widespread emergence of antibiotic resistant mastitis pathogens as a result of antibiotic use on farms. Another example where antibiotic treatment does not appear to cause emergence or amplification of antibiotic resistance is by Singer et al (87). In that study, ceftiofur treated and untreated cattle were examined to document the genetic diversity of *E.coli* isolates from these cattle. Ceftiofur resistant *E.coli* were only isolated from treated cows during and immediately following the cessation of treatment but did not show an increase in numbers within treated cows or long term persistence in those cows. In another study by Erskine et al (33), proportions of resistant *S. aureus* from Michigan dairy herds did not show an increase in resistance to antibiotics, however, certain microorganism–antibiotic combinations recorded an increase in resistance such as *S. uberis* resistant to penicillin.

Rajala Schulz et al. (80) studied the antimicrobial susceptibility of mastitis pathogens isolated over a 16-month period from first lactation and older cows. Target

organisms studied include CNS, esculin positive streptococci and Gram-negative pathogens. They observed no differences in the MICs of *streptococci* and Gram-negative pathogens, however CNS isolates showed penicillin and tetracycline resistance in both first lactation and older cows indicating antibiotic use is a main factor in development of resistant organisms.

CNS are one of the most common cause of mastitis and believed to serve as a pool of antibiotic resistance genes that can be transmitted to *S. aureus* which may result in the emergence of MRSA or MDR MSSA. Sawant et al. (82) analyzed the antibiotic susceptibility profiles of CNS from three dairy research farms in 2005 and reported that a majority of these were susceptible to ampicillin, oxacillin, cephapirin, ceftiofur, erythromycin, pirlimycin. The exceptions to this were *S. epidermidis* isolates which were resistant to erythromycin and pirlimycin.

Another way to assess the effect of antibiotic use on the emergence of resistant organisms is to compare and contrast farm systems which employ different production strategies such as organic diaries (which use little or no antibiotics) and conventional dairies (which regularly use antibiotics in the prevention and treatment diseases) (68). Poll and Ruegg (75) compared the antibiotic susceptibility of bacteria between organic and conventional dairies from 1994 to 2000. All target microoganisms (CNS, *Streptococcus spp., S. aureus, S. agalactiae*) except coliforms were more prevalent on organic herds. They found that farm type was associated with the MIC of ampicillin and tetracycline for CNS and with pirlimycin and tetracycline for *streptococcus spp.* However, *S. aureus* isolates exhibited heterogeneity in MIC value which was likely

dependent on the amount of exposure to penicillin and pirlimycin.

Antimicrobial resistant bacteria become a significant public health risk when they are able to establish themselves in an environment and then clonally disseminate (such as HA MRSA). However, it does not appear that antibiotic resistant mastitis pathogens, particularly antibiotic resistant *S. aureus* such as MDR MSSA (17.2% of bulk tank samples) or MRSA (1.3% of bulk tank samples), have become well established in the dairy environment nor show clonal dissemination within the dairy environment. Although the presence of MDR MSSA tended to be more likely in herds that used tetracyclines, phenicols and routinely used DCT, this relationship was not significant.

Since no previous studies have analyzed the relationship between farm management factors and the occurrence of MDR *S. aureus* or MRSA, we report the first such statistical modeling of farm practices and farm-level factors. Northwest and Southeast regions of MN were observed to have lower risk for the presence of MDR MSSA in bulk tank milk, as compared to Southwest region ($P<0.05$). There is no obvious or immediate explanation for this relationship. Similarly, the risk for finding MDR MSSA in bulk tank milk was increased in closed herds. Again, there is no obvious explanation for this relationship. While previous studies have analyzed the relationship between antibiotic use (as no usage, low usage and high usage) (75) and corresponding resistance (measured in terms of MICs) in pathogens, specific patterns of antibiotic use such as the use of certain classes of antibiotic for treatment of specific diseases have not been described till date. Further larger scale and more in depth studies would need to be conducted to investigate the potential underlying factors associated with these relationships. Such studies might

potentially include collecting soil and water samples, and samples from farmers and veterinarians in addition to samples of bovine origin. It may also be interesting to investigate farm workers, their family occupations and environments they come into contact with on a regular basis. Such future studies will help to identify particular situations where infection and/or contamination of the dairy cows or milk, respectively, may be occurring. Such analysis can assist farmers and dairy veterinarians in decision-making regarding appropriate management practices to be adopted. This study also serves to provide information for case-control studies to monitor the situation of antibiotic resistant pathogens on dairy farms.

Also, this is the first study to model herd level factors for the presence of MRSA in BTM in dairies. One reason we did not detect any significant associations could be due to the small number of resistance events (2 of 150) which constituted the dependent variable.

Thus, future studies enrolling more herds over a longer period may provide more power to detect certain associations. Also, appropriate case control studies with antibiotic exposure in one group and no exposure in the other will help evaluate if exposure to antibiotics has significant impact on the detection of MDR MSSA and MRSA in dairies.

In conclusion the current study did not identify any farm-level or herd management factors associated with the presence of MRSA in bulk tank milk. The risk for presence of MDR MSSA in bulk tank milk was increased in herds from the SW region of MN (vs NW or SE region) and in closed (vs. open) herds. Future studies are needed to confirm the trends identified in this study: use of phenicols, with decreased risk of finding MDR

MSSA and use of DCT associated with an increase in risk of finding MDR MSSA in BTM, as well as to identify other associations which may not have been detected due to the limited number of herds enrolled in this study. Future studies should be designed to understand the relationship between the use of different classes of antimicrobials and detection of resistance in zoonotic pathogens such as *S. aureus*.

Table IV-1: Classification of commercial antibiotics used in the 50 MN dairies according to the antibiotic classes of their active compounds.

Antibiotic	Compound	Antibiotic Class
Dry cow intramammary antibiotic products used at dry off:		
Albadry/ Novodry	Penicillin&Novobiocin	Penicillin/Aminocoumarin
Biodry	Novobiocin sodium	Aminocoumarin
Cefa-Dri	Cephapirin benzathine	1st Gen Cephalosporin
Gally/ ErythroDry	Erythromycin	Macrolide
Go-Dry	Procaine Penicillin G	Beta Lactam
Quartermaster	Penicillin dihydrostreptomycin	Penicillin/Aminocoumarin
Tomorrow	Cephapirin	1st Gen Cephalosporin
Orbenin DC	Benzathine cloxacillin	Beta Lactam
Spectramast DC	Ceftiofur hydrochloride	3rd Gen Cephalosporin
Lactating cow intramammary antibiotic products used to treat CM in lactating cows		
Aquamast	Penicillin G	Beta Lactam
Cefa-Lak	Cephapirin Sodium	1st Gen Cephalosporin
Dariclox	Sodium Cloxacillin	Beta Lactam
Gallimycin 35	Erythromycin	Macrolide
Mast-Clear	Procaine Penicillin G	Beta Lactam
Pirsue	Pirlimycin HCl	Lincosamide
ToDAY	Cephapirin sodium	1st Gen Cephalosporin
Spectramast LC	Ceftiofur hydrochloride	3rd Gen Cephalosporin
Systemic products used to treat clinically ill adult cows		
Ceftiofur	Ceftiofur	3rd Gen Cephalosporin
Tetracycline	Tetracycline	Tetracycline
Penicillin	Penicillin	Beta Lactam
Ampicillin	Ampicillin	Beta Lactam
Erythromycin	Erythromycin	Macrolide
Nuflor	Florfenicol	Phenicol
Tylan 200	Tylosin	Macrolide
Tylsin	Tylosin	Macrolide
Micotil	Tilmicosin	Macrolide
Draxxin	Tulathromycin	Macrolide

Table IV-2: Herd Statistics for 50 MN study herds.

	Mean	SD	Min	Max
Number of milking cows	270.9	333.7	43	1592
Total number of cattle	580.6	688.1	89	3320
Rolling herd average (lbs. milk/cow/year)	21586.1	4241	8769	28162
Daily milk yield (lbs milk/cow/day	74	16.2	11	95

Table IV-3: Herd Size Characteristics for 50 MN study herds.

	Frequency observed	%
Category of herd size		
40-99	15	30
100-299	19	38
>300	16	32
Distribution of herds by region		
NW	16	32
SE	18	36
SW	16	32
Distribution of breeds among herds		
Brown Swiss	1	2
Holstein	49	98
Herd Status as closed or open		
Closed	26	52
Open	23	46
Herd status as organic or conventional		
Organic	0	0
Conventional	50	100

Table IV-4: Production Characteristics for 50 MN study herds.

	Frequency observed	%
Average bulk tank somatic cell count		
<10,000	1	2
100,000 - 199,000	16	32
200,000 - 299,000	20	40
300,000 - 399,000	11	22
400,000 - 499,000	1	2
500,000 +	1	2
Average bacterial counts		
0 - 4,999	25	50
5,000 - 9,999	18	36
10,000 - 19,999	3	6
20,000 - 29,999	3	6
30,000 - 39,999	1	2
Consumption of unpasteurized milk from the operation		
Consumed	18	36
Consumed after home pasteurization	6	12
Not consumed	26	52
Milk or meat residue violation in previous year		
No	46	92
Yes	4	8

Table IV-5: Classification of Bedding and Sick cow Housing among 50 MN study herds.

	Frequency observed	%
Isolated sick cow housing		
Yes	11	22
No	39	78
Types of bedding for lactating cows		
Dried manure	0	0
Other organic bedding	31	62
Inorganic bedding	10	20
Dried manure and other organic bedding	2	4
Inorganic and other organic bedding	7	14

Table IV-6: Percentage of cows receiving dry cow therapy (DCT) among the 50 MN study herds.

	Frequency observed	%
% cows receiving DCT		
0	3	6
10	1	2
12	1	2
100	45	90

Table IV-7: Health Management and antibiotic use classification for the 50 MN study herds.

Classes of Antibiotics used	IMM Ab use in DCT		IMM Ab use in CM		Systemic Ab use to treat								Total Ab use on farm	
					CM		RD		UI		Lameness			
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Penicillin/Aminocoumarin	20	40	-	-	-	-	-	-	-	-	-	-	20	40
1st Gen Cepalosporins	36	72	36	72	-	-	-	-	-	-	-	-	47	94
Macrolide	-	-	-	-	6	12	6	12	-	-	-	-	10	20
Beta-lactams	2	4	1	2	24	48	11	22	18	36	17	34	40	80
3rd Gen Cephalosporins	4	8	37	74	14	28	25	50	22	44	23	46	45	90
Aminocoumarins	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Phenicols	30	60	-	-	1	2	5	10	-	-	-	-	5	10
Tetracyclines	-	-	-	-	16	32	6	12	19	38	14	28	36	72
Lincosamides	-	-	27	54	-	-	-	-	-	-	-	-	27	54
Other organic/ natural	-	-	12	24	5	10	2	4	-	-	-	-	16	32
Anti-inflammatory products	-	-	-	-	29	58	20	40	12	24	22	44	37	74

*Ab- antibiotic,
CM-Clinical mastitis
RD-Respiratory disease
UI-uterine infections or metritis
Gen- generation

Table IV-8: Prevalence and resistance characteristics of MSSA isolates from BTM of 50 MN study herds.

Characteristic	No. (%) of Isolates	Total no. of samples
Total prevalence of MSSA isolates	93 (62 %)	150
Resistant to at least one class of Ab	38 (40.8%)	150
Resistant to at least two classes of Ab (MDR)	16 (17.2%)	150
Resistant to 0 classes of Ab	55 (59.1 %)	150
Resistant to 1 class of Ab	22 (23.6%)	150
Resistant to 2 classes of Ab	11 (11.8%)	150
Resistant to 3 classes of Ab	1 (1%)	150
Resistant to 4 classes of Ab	2 (2.1%)	150
Resistant to 5 classes of Ab	2 (2.1%)	150

*Ab stands for antibiotic
** MDR refers to multidrug resistant isolates.

Table IV-9: Classification of Class of Drug Resistance for MSSA isolates from 50 MN Dairy Herds (total = 93 isolates).

Antibiotic Class Isolate is Resistant to	No. of resistant isolates
Beta-lactams	32 (34.4 %)
1st generation Cephalosporin	1(1%)
3rd generation Cephalosporin	1 (1%)
Macrolide	7 (7.5%)
Aminocoumarin	4 (4.3%)
Lincosamides	4 (4.3%)
Aminoglycosides	3 (3.2%)
Tetracyclines	12 (12.9%)

Table IV-10: Factors identified to be associated on univariate analysis at P<0.3 for the occurrence of MRSA in bulk tank milk

Variable	Estimate (SE)	P	Type3 P-value
Residue Violation in past year			
No	0.0142 (0.0098)	0.1485	0.2556
Yes	ref	.	
Use of DCT			
Little	-0.0148 (0.0102)	0.1482	0.2114
100%	ref	.	
Use of β-lactam/aminoucomarins for IM dry cow therapy			
No	0.0222 (0.0152)	0.1430	0.1527
Yes	ref	.	
Use of 1st generation cephalosporins for IMM dry cow therapy			
No	-0.1183 (0.0126)	0.1456	0.1633
Yes	ref	.	
Use of β-lactam for IMM dry cow therapy			
No	0.0140 (0.0097)	0.1485	0.2459
Yes	ref	.	
Use of 3rd gen cephalosporins for IMM dry cow therapy			
No	0.0145 (0.0100)	0.1481	0.2265
Yes	ref	.	
Use of 1st generation cephalosporins for systemic treatment of CM			
No	-0.0185 (0.0127)	0.1456	0.1610
Yes	ref	.	
Use of 3rd gen cephalosporins for IMM treatment of CM			
No	-0.0179 (0.0123)	0.1462	0.1647
Yes	ref	.	
Use of other organic/inorganic products for IM treatment of CM			

No	0.0175 (0.0121)	0.1465	0.1670
Yes	ref	.	
Use of tetracyclines for IMM treatment of CM			
No	0.0196 (0.0135)	0.1452	0.1585
Yes	ref	.	
Use of anti-inflammatory drugs for systemic treatment of CM			
No	0.0317 (0.0214)	0.1371	0.1467
Yes	ref	.	
Use of other organic/inorganic products for IMM dry cow therapy			
No	0.0148 (0.0148)	0.1482	0.2114
Yes	ref	.	
Use of 3rd generation cephalosporins for systemic treatment of UI			
No	0.0241 (0.0164)	0.1417	0.1504
Yes	ref		
Use of β-lactams for systemic treatment of UI			
No	0.0208 (0.0143)	0.1441	0.1559
Yes	ref	.	
Use of anti-inflammatory drugs for systemic treatment of UI			
No	0.0175 (0.0121)	0.1465	0.1670
Yes	ref	.	
Use of tetracyclines for systemic treatment of RD			
No	0.0152 (0.0105)	0.1480	0.1993
Yes	ref		
Use of anti-inflammatory drugs for systemic treatment of RD			
No	-0.0333 (0.0224)	0.1360	0.1462
Yes	ref	.	
Use of other organic/inorganic products for systemic treatment of RD			
No	0.0139 (0.0096)	0.1487	0.3024
Yes	ref	.	

Use of tetracyclines for systemic treatment of lameness No Yes	 0.0185 (0.0127) ref	 0.1459	 0.1622
Use of 3rd generation cephalosporins for systemic treatment of lameness No Yes	 -0.0290 ref	 0.1389	 0.1478
Overall use of β-lactams on farm No Yes	 0.0222 (0.0152) ref	 0.1430	 0.1527
Overall use of 1st generation cephalosporins on farm No Yes	 -0.0142 (0.0098) ref	 0.1484 .	 0.2390
Overall use of 3rd generation cephalosporins on farm No Yes	 -0.0148 (0.0102) ref	 0.1482 .	 0.2114
Overall use of anti-inflammatory drugs on farm No Yes	 -0.0180 (0.0124) ref	 0.1462	 0.1644

Ref = referent class of categorical variable
IMM= Intra mammary
DCT= Dry Cow Therapy
CM= Clinical mastitis
UI= Uterine infections or metritis
RD= Respiratory diseases

Table IV-11: Factors identified to be associated on univariate analysis at P<0.2 for the occurrence of MDR MSSA in bulk tank milk.

Variable	Estimate	P	Overall/Type3 P-value
Region			0.0225
NW	-0.000(0)	.	
SE	0.1370(0.0247)	0.1336	
SW	ref	.	
Herd Size	0.0000 (0.0000)	0.5877	0.1225
No. of milk cows	0.0001 (0.0001)	0.2794	0.1267
Residue Violation			
No	0.0142 (0.0098)	0.1485	0.1027
Yes	ref	.	
Use of DCT			
None or little	-0.0148 (0.0102)	0.1482	0.0422
100%	ref	.	
Herd Status			
Closed	-0.0021	0.9083	0.1653
Open	ref	.	
Use of 3rd gen cephalosporins for systemic treatment of CM			
No	-0.0146 (0.0247)	0.5557	0.1518
Yes	ref	.	
Use of tetracyclines for systemic treatment of CM			
No	0.0196 (0.0135)	0.1452	0.0884
Yes	ref	.	
Use of tetracyclines for systemic treatment of RD			
No	0.0196 (0.0135)	0.1452	0.0884
Yes	ref	.	
Use of macrolides for systemic treatment of RD			
No	-0.0450	0.3557	0.0234

Yes	ref	.	
Use of phenicols for systemic treatment of RD			
No	-0.0550 (0.0565)	0.3304	0.0338
Yes	ref	.	
Use of anti-inflammatory drugs for systemic treatment of RD			
No	-0.0333 (0.0224)	0.1360	0.1118
Yes	ref	.	
Use of organic/natural products for systemic treatment of RD			
No	0.0139 (0.0139)	0.1487	0.1745
Yes	ref		
Use of 3rd generation cephalosporins for systemic treatment of lameness			
No	-0.0290 (0.0196)	0.1389	0.1575
Yes	ref	.	
Overall use of macrolides for treatment of any infection/disease			
No	-0.0250 (0.0327)	0.4442	0.1048
Yes	ref	.	
Overall use of tetracyclines for treatment of any infection/disease			
No	0.0146 (0.0247)	0.5555	0.1225
Yes	ref		
Overall use of phenicols for treatment of any infection/disease			
No	-0.0667 (0.601)	0.3239	0.0422
Yes	ref	.	

Ref = referent class of categorical variable
'Herd size' variable defines the total number of cattle on farm including milk cows
IMM= Intra mammary
DCT= Dry Cow Therapy
CM= Clinical mastitis
UI= Uterine infections or metritis
RD= Respiratory diseases

Table IV-12: Generalized estimating equations with binary outcome of factors significantly (P<0.05) associated with the occurrence of MDR *S. aureus* in BTM of 50 MN dairy farms.

Table IV-12a) GEE outcome for model I: All diseases model

Variable	Estimate (SE)	P-value	O.R (95% C.L)
Intercept	0.1418 (0.0775)	0.0673	1.1523(1.0664, 1.341)
Region		*0.0113	
NW	-0.2630 (0.0727)	0.0003	0.7687 (0.6667, 0.8863)
SE	-0.2411(0.0725)	0.0009	0.7857 (0.6816, 0.9057)
SW	ref	.	0
Herd Status		*0.0543	
Closed	0.0998 (0.0498)	0.0451	1.1049 (1.0022, 1.2182)
Open	ref	.	0
Use of Phenicols		*0.1229	
No	0.0911 (0.0442)	0.0395	1.0953 (1.0499, 1.1428)
Yes	ref	0	0

* Type 3 P-value
Ref = referent class of categorical variable

Table IV-12 b) Generalized estimating equations with binary outcome of factors significantly (P<0.05) associated with the occurrence of MDR *S. aureus* in BTM of 50 MN dairy farms; GEE outcome for model II: Mastitis Model

Variable	Estimate (SE)	P-value	O.R (95% C.L)
Intercept	0.2330 (0.0727)	0.0014	1.2623 (1.0947, 1.4557)
Region		*0.0115	
NW	-0.2575 (0.0718)	0.0003	0.7729 (0.6715, 0.8877)
SE	-0.2461 (0.0735)	0.0008	0.7818 (0.6769,0.9029)
SW	ref	.	0
Use of Dry Cow Therapy		*0.1244	
None or Little	-0.0888 (0.0449)	0.0478	0.9150 (0.8379, 0.9857)
100%	ref	.	0
Herd Status		*0.0552	
Closed	0.0992 (0.0496)	0.0457	1.1042 (1.0019, 1.2170)
Open	ref	.	0

* Type 3 P-value
Ref = referent class of categorical variable

Table IV-12 c) Generalized estimating equations with binary outcome of factors significantly (P<0.05) associated with the occurrence of MDR *S. aureus* in BTM of 50 MN dairy farms; GEE outcome for model III: Other diseases model

Variable	Estimate (SE)	P-Value	O.R (95 % C.L)
Intercept	0.1482(0.0768)	0.0536	1.1597(1.0023, 1.3481)
Region		*0.0113	
NW	-0.2631 (0.0727)	<0.0003	0.7686 (0.6655, 0.8862)
SE	-0.2397 (0.0725)	<0.0009	0.7868 (0.6826, 0.9070)
SW	ref	.	0
Herd Status		*0.0567	
Closed	0.0984 (0.0496)	0.0473	1.103 (1.0012, 1.2160)
Open	ref	.	0
Use of Phenicols		*0.1266	
No	0.0849 (0.0423)	0.0446	1.088 (1.0020, 1.1827)
Yes	ref	.	0

* Type 3 P-value
Ref = referent class of categorical variable

Table IV-13: List of 70 independent variables analyzed for statistical associations with the presence of MDR MSSA and MRSA in BTM in MN dairies.

Herd Id	**Systemic use of macrolides for CM**
Region	**Systemic use of phenicols for CM**
Season	**Systemic use of anti-inflammatory products for CM**
Total number of cattle (herd size)	**Systemic use of other organic/inorganic products for CM**
Total number of milk cows	**Systemic use of 3rd generation cephalosporins for UI**
Category of herd size as small, medium or large	**Systemic use of tetracyclines for UI**
Somatic cell count in milk	**Systemic use of beta-lactams for UI**
Bacterial counts in milk	**Systemic use of macrolides for UI**
Organic or conventional status of herd	**Systemic use of phenicols for UI**
Cow breed of herd	**Systemic use of anti-inflammatory products for UI**
Bedding type as organic, inorganic or combination of two for lactating cows	**Systemic use of other organic/inorganic products for UI**
Use of DCT	**Systemic use of 3rd generation cephalosporins for RD**
Herd status as closed or open	**Systemic use of tetracycines for RD**
Use of rBST	**Systemic use of beta-lactams for RD**
Residue violation in the past year	**Systemic use of macrolides for RD**
IMM use of Penicillin/Novobiocin for DCT	**Systemic use of phenicols for RD**
Use of 1st generation cephalosporins for DCT	**Systemic use of anti-inflammatory products for RD**
Use of macrolides for DCT	**Systemic use of other organic/inorganic products for RD**
Use of beta-lactams for DCT	**Systemic use of 3rd generation cephalosporins for lameness**
Use of 3rd generation cephalosporins for DCT	**Systemic use of tetracyclines for lameness**
Use of aminocoumarins for DCT	**Systemic use of beta-lactams for lameness**
Use of Orbeseal for DCT	**Systemic use of anti-inflammatory products for lameness**
Use of other organic/inorganic products for DCT	**Overall use of penicillin/novobiocin to treat all diseases on farm**
IMM Use of 1st generation cephalosporins for CM	**Overall use of 1st generation cephalosporins to treat all diseases on**

	farm
IMM Use of beta-lactams for CM	**Overall use of macrolides to treat all diseases on farm**
IMM Use of macrolides for CM	**Overall use of beta-lactams to treat all diseases on farm**
IMM Use of 3rd generation cephalosporins for CM	**Overall use of third generation cephalosporins to treat all diseases on farm**
IMM Use of lincosamides for CM	**Overall use of aminocoumarins to treat all diseases on farm**
IMM Use of other organic/inorganic products for CM	**Overall use of lincosamides to treat all diseases on farm**
Systemic use of 3rd generation cephalosporins for CM	**Overall use of tetracyclines to treat all diseases on farm**
Systemic use of tetracyclines for CM	**Overall use of phenicols to treat all diseases on farm**
Systemic use of beta-lactams for CM	**Overall use of anti-inflammatory products to treat all diseases on farm**

*Abbreviations used in above table refer to:
DCT=Dry cow Therapy
rBST= Recombinant bovine somatotropin
IMM= Intra mammary
CM= Clinical Mastitis
RD= Respiratory Diseases
UI= Uterine Infections or metritis

PRODUCER CONSENT FORM

Development and Application of a Minnesota Dairy Health Surveillance Network - Epidemiology of *Escherichia coli* in MN dairy calves and *Staphylococcus aureus* in MN bulk tank milk

The Department of Veterinary Population Medicine, College of Veterinary Medicine at the University of Minnesota is conducting a study to describe the epidemiology of *Escherichia coli* in Minnesota dairy calves, with emphasis on Shiga toxin producing *E. coli* (STEC), and to describe the epidemiology of *Staphylococcus aureus* in bulk tank milk samples.

Project Goal: Infectious pathogens in food animal production systems are of great significance to food animals, producers, and consumers, as well as to local and national economies. By implementing an on-going surveillance program we can improve our understanding of the possible relationships between herd management practices and issues of food safety or public health concern, or describe trends in disease rates and agents over time, or to identify when important changes in existing pathogens occur. This information may be used to develop strategies to limit their impact on animals and producers, and to reduce or eliminate risks to consumers through the food supply. Programs must also be in place to rapidly detect and react to new (foreign, emerging or introduced) infectious disease threats, to detect new virulence patterns in existing pathogens, or to detect shifts in antimicrobial resistance patterns for pathogens of concern to both animal and public health.

The ***objective of this project*** is to establish a prospective surveillance program to:

1. Describe the prevalence and genetic diversity of *Staphylococcus aureus* in bulk tank milk and
2. Describe the prevalence and genetic diversity of *Escherichia coli* in feces of calves.

Project Activities: Fifty Minnesota dairy herds will be enrolled in the study. A study technician will visit the herd three times between January and December, 2009. At each of these three visits, the study technician will collect 5 fresh fecal samples from the pen environment of the post-weaned calves (ages approximately 8 to 16 weeks). These samples will be transported back to the University of Minnesota laboratory for culture and further analysis of any *E. coli* bacteria isolated from the fecal samples collected. The

technician will take appropriate biosecurity cautions (clean coveralls, disinfected boots, clean disposable gloves) when on the farm.

At the same visit the technician will pick up frozen bulk tank milk samples, previously collected by the producer. These milk samples will be transported back to the University of Minnesota udder health laboratory for routine bulk tank culture and further analysis of any *S. aureus* isolated from bulk tank samples. All costs associated with the study will be covered by the University of Minnesota (e.g. travel, supplies, laboratory fees).
As part of this study, I agree to allow the study technician to visit the farm to collect the necessary fecal samples. I also agree to keep records of all calfhood disease treatment and mortality events throughout the study period (February, 2009 to August, 2010). Finally, I agree to complete a questionnaire describing herd management practices for calves (e.g. housing, nutrition program, treatment protocols) three times during the study, coinciding with each of the three sampling events over the 12 month study.

I recognize that the risks associated with collection of fecal samples from the calf environment bears a minimal risk. I will be financially compensated with $100 for each time that I complete the herd management questionnaire ($300 over the 12 month study period). I will receive bulk tank culture results. Also, at the conclusion of the study I understand that I will receive a summary report of study findings.

I acknowledge that my participation in this project is voluntary and that I can withdraw from the study at any time. However, I agree to discuss any concerns to the principle investigators in an attempt to resolve any potential problems prior to the occurrence of such a withdrawal. In case of any problems, questions or concerns with the study, you may contact:

Dr. Sandra Godden, the principal investigator: 612-625-8177

CONSENT

I, _____ , have read and understand the description of this study, and agree to participate.

CLIENT NAME

_____ DATE_____

PRINCIPAL INVESTIGATORS' NAMES (contact persons if questions or problems arise)

_____ DATE _____

Sandra Godden 612-625-8177

University of Minnesota Institutional Animal Care and Use Committee 612-626-5654

MN Dairy Health Surveillance Study – HERD MANAGEMENT QUESTIONNAIRE

(Spring, 2009)

Explanation: Please find enclosed a producer consent form and a herd management questionnaire. Please sign the consent form and complete the following questionnaire to the best of your ability. If you are uncertain about how to answer some questions, please contact Dr. Sandra Godden at 612-625-8177. This first questionnaire will be comprehensive. A second and third questionnaire, to be administered in the summer and fall sampling events, will be considerably shorter. You will be compensated $100 each, for completing each of the questionnaires.

Once completed, please mail the signed consent form and completed questionnaire back to Sandra Godden in the self-addressed stamped envelope provided.

Again, thank you very much for your participation on this project.

Sincerely,

Sandra Godden Tel: 612-625-8177

A. PRODUCER INFORMATION

Farm name:_____

Owner(s) name:_____

Contact person or herdsman
(if different from owner):_____

Herd Veterinarian:_____

DHIA Herd Number:_____

DHIA Access code:_____

Grade A permit number: _____

Federal Tax ID # (to pay farm for completing questionnaire): _____

Person completing questionnaire:_____

Today's Date: _____

B. INVENTORY – HERD SIZE / EXPANSION STATUS

B.1. As of today, what is your approximate inventory of the following groups of cattle?

	Lactation 1*	Lactation 2 & up*	Total
A. Milking cows			
B. Dry cows			
C. Total cows (add totals of A. and B. above)			
D. Preweaned (milk-fed) heifer calves			
E. Weaned replacement calves and heifers*			
F. Other youngstock (e.g. steers)			

G. Bulls **		
H. Total cattle (Add C-G above)		

* "Weaned replacement calves and heifers" here means all female animals that will be kept as replacement cows, and have not yet calved yet.

** Include only bulls kept for breeding purposes (e.g., breeding age bulls or younger bulls being saved for breeding purposes)

B.2. During the past 12 months were any of the following groups of animals brought onto this operation from outside sources?

	Brought onto operation?	If YES, How many?
A. Preweaned calves?	□Yes □No	
B. Heifers weaned to breeding age?	□Yes □No	
C. Heifers breeding age to 1st calving?	□Yes □No	
D. Dairy cows?	□Yes □No	
E. Bulls?	□Yes □No	
F. Other cattle (include beef)?	□Yes □No	
E. Total		

B.3. In the past 12 months, have you taken cattle (calves, heifers, cows, bulls) to public shows (e.g. fairs) where they were in contact with other cattle, then returned them to the farm?

Yes_____ No_____

B.4. This question refers to animals **other than dairy cattle** on this operation.

Within the last 12 months, have any of the following types of animals been present on this operation? If so, please indicate whether these animals had physical contact* with any of this operation's dairy cows or heifers, or their feed, minerals, or water supply.

	Present on operation?	Physical contact* with cattle?
A. Beef cattle?	☐ Yes ☐ No	☐ Yes ☐ No
B. Chickens, turkeys, domestic geese, or other poultry?	☐ Yes ☐ No	☐ Yes ☐ No

C. Horses or other equines	☐ Yes ☐ No	☐ Yes ☐ No
D. Pigs?	☐ Yes ☐ No	☐ Yes ☐ No
E. Sheep?	☐ Yes ☐ No	☐ Yes ☐ No
F. Goats?	☐ Yes ☐ No	☐ Yes ☐ No
G. Farmed exotic animals (such as elk, llamas, ostriches, etc)? Specify:_____	☐ Yes ☐ No	☐ Yes ☐ No
H. Dogs?	☐ Yes ☐ No	☐ Yes ☐ No
I. Cats?	☐ Yes ☐ No	☐ Yes ☐ No
J. Wild geese, other wild birds?	☐ Yes ☐ No	☐ Yes ☐ No

* As used here, "**physical contact**" means nose-to-nose contact, including through a fence, or shared feed and water resources.

C. PRODUCTION

C.1. Is this herd certified as a USDA organic producer?(circle one)

A. Yes, the farm has been certified organic for _____years
B. No, but the farm is in the process of applying for organic certification
C. No, this is a conventional herd

C.2. The predominant breed of cow on the dairy is: _____
(e.g. Holstein, Jersey, cross-bred, other…)

C.3. During the last 6 months, which of the following best describes the average bulk tank
somatic cell count (cells/ml) for milk shipped? (Circle one).

A. < 100,000
B. 100,000 – 199,000
C. 200,000 – 299,000
D. 300,000 – 399,000
E. 400,000 – 499,000
F. 500,000 +

C.4. During the last 6 months, which of the following best describes the average bacterial
count (SPC, plate loop count) for milk shipped? (Circle one).

A. 0 – 4,999
B. 5,000 – 9,999
C. 10,000 – 19,999
D. 20,000 – 29,999
E. 30,000 – 39,999
F. 40,000 +

C.5. What is current milk per cow per day? _____lbs/cow/day

C.6. Does the herd use rBST (Posilac) in lactating cows? Yes _____ No_____

C.7. Is unpasteurized milk that is produced on this operation consumed by family members, farm workers, or others?

1. Unpasteurized milk from this operation is consumed.
2. Home pasteurizer is used for milk produced on this operation.
3. Unpasteurized milk is not consumed. All milk consumed is purchased

C.8. Has the herd had any milk or meat residue violations in the past year?

Yes _____ No_____

If yes, please describe:
_____

D. HOUSING

D.1. Which one of the following types of milking facilities did this operation primarily use during the past 12 months? (Circle one)

A. Parallel or herringbone parlor?
B. Flat parlor or step-up parlor milking facility?
C. Tie Stall or stanchion barn pipeline facility?
D. Rotary parlor?
E. Other type of milking facility?
(specify)_____

D.2. What housing facilities did this farm use in the past 12 months (check all that apply):

	Hutch (single calf only)	Individual pen in calf barn	Calf is tied in tie stall barn	Group housing	Free-stall	Tie Stall	Bedded pack	Pasture
A. Preweaned (milk-fed) dairy calves?								
B. Weaned dairy calves & heifers?								
C. Lactating dairy cows?								
D. Maternity housing (where cows								

D.3. Is Maternity housing (where cows normally calve) in a separate pen or facility from other lactating cows?.....

Yes_____ No_____

D.4. Sick cows are most often housed…(circle one)

A. In maternity pens
B. In pens with fresh cows other
C. Remain In their original pen with other cows
D. In a separate pen, stall or facility from cows

D.5. Sick preweaned calves are most often housed...(circle one)

A. Are not moved out of their original pen
B. Are isolated by moving away from direct contact with healthy calves

D.6. Sick postweaned calves are most often housed...(circle one)

A. Are not moved out of their original pen
B. Are isolated by moving away from direct contact with healthy calves

E. FEED and WATER – ADULT COWS

E.1. Do you feed a total mixed ration (TMR) to lactating dairy cows?
Yes_____ No_____

E.2. In the last 60 days, which of the following feeds have been used in rations for the following groups of dairy animals? (check all that apply)

Type of Feed	Lactating or Dry Cows	Bred heifers	Postweaned heifers
A. Whole cottonseed/hulls			
B. Whole soybeans or soybean meal			
C. Bakery by-products			
D. Brewers by-products (brewers or distillers' grains)			
F. Blood meal			
G. Meat & bone meal			
G. Milk products (e.g., whey)			
H. Tallow/animal fat			
I. Other protein meal (e.g., meal from fish or poultry) Please specify:_____			

E.3. Is the ration for close-up dry cows different from the ration for far-off dry cows?
Yes_____ No_____

E.4. Do you normally feed anionic salts (DCAD diet) to close-up transition cows?
Yes_____ No_____

E.5. Are cows fed Rumensin (monensin) in the transition and lactating cow rations?
Yes_____ No_____

E.6. What is the source of drinking water for cattle?

A. Well B. Surface water
C. Municipal water D. Other (please specify)_____

E.7 Is the water that dairy cattle drink usually chlorinated?
Yes_____ No_____

E.8. How often are water tanks or buckets cleaned for the following groups?

	Frequency Cleaned	Frequency Disinfected	Name disinfectant used
Milking cows	_____times/week	_____times/week	
Dry cows	_____times/week	_____times/week	
Bred heifers	_____times/week	_____times/week	
Postweaned heifers	_____times/week	_____times/week	
Preweaned heifers	_____times/week	_____times/week	

F. BEDDING

F.1. Which of the following bedding types are typically used for the following groups of animals, and how often is this bedding changed or added to?

	For each bedding type, put a number 1-7 (select from list below) corresponding to how often the bedding is changed or added to)		
	Dried manure	Other organic bedding*	Inorganic bedding**
Lactating cows			
Maternity, close-up, or recently fresh cows			
Sick cows			
Preweaned (milk-fed) calves			
Postweaned calves/bred heifers			

1. Daily
2. Every 2-3 days.
3. Weekly (more than 3 days, less than 8 days)
4. 2-3 times per month
5. Monthly
6. Greater than monthly

* "Organic bedding" includes straw, sawdust, newspaper, corn cobs or stalks, excluding dried manure.

** "Inorganic bedding" includes sand, rubber tires, mats, mattresses, crushed limestone

G. MANURE MANAGEMENT

G.1. Are any of the following waste **storage** systems used on this operation? (Circle all that apply)

1. Below floor or deep pit
2. Anaerobic lagoon with cover
3. Slurry storage in earth-basin
4. Anaerobic lagoon without cover
5. Slurry storage in Slurrystore® (or similar storage structure)
6. Aerated lagoon
7. Manure pack (inside barn)
8. Outside storage within dry lot or pens
9. Outside storage for solid manure not in dry lot or pen
10. Storage of solid manure in a building without cattle access

G.2. You may respond to this question in miles or feet. What is the distance between the manure storage area and the nearest of the following: (Put "N/A" if not applicable—e.g., if use municipal water and no well is nearby)

A. Well*? _____miles or _____feet

B. Waterway or body of water Accessible by animals? _____miles or _____feet

* If more than one manure storage area is used, include only the one closest to the well or body of water.

G.3. Which of the following methods are used to dispose of manure on owned or rented land? (Circle all that apply)

1. Irrigation
2. Slurry (surface application)
3. Broadcast/solid spreader
4. Slurry (subsurface application)
5. Other method (specify)_____

6. Do not apply manure on owned or rented land.

G.4. Do you use separate loader buckets for moving feed and for handling manure? (Circle the appropriate number 1-3)

1. Yes, use separate buckets.
2. No, do not use separate buckets.
3. Do not use this equipment for handling manure.

If 2. is circled, answer G.5.

G.5. After you have used the loader bucket for handling manure, do you do any of the following before using it for feed?: (Circle the appropriate number 1-4)

1. Rinse bucket with water only.
2. Power wash bucket with high pressure water.
3. Wash and disinfect bucket. → List disinfectant_____
4. Do not wash or disinfect bucket

H. CALF MANAGEMENT and FEEDING

H.1. How long do newborn heifer calves stay in the maternity pen?

_____ hrs after birth or _____ days after birth

H.2. After removing from the maternity pen, newborn calves are moved to housing (circle one):
A. On the same farm site as the milking herd
B. To a different farm site

H.3. Which one of the following methods is used most frequently for the first feeding of colostrum to newborn dairy heifer calves?

A. Calf is left to nurse the cow for a period of time (e.g. > 2-4 hours)

B. Hand feeding from bucket or bottle

C. Hand feeding using esophageal tube feeder

D. Fed a commercial colostrum replacer. Product name:_____

E. Do not get colostrum

H.4. If feeding maternal colostrum, is it (circle one):

A. From one cow only

B. Pooled colostrum (mixed from more than one cow)

H.5. If feeding maternal colostrum, is it (circle one):

A. Raw colostrum

B. Pasteurized colostrum

H.6. If colostrum is hand fed (bucket, bottle or tube), how long after birth are most calves
provided the first feeding?

_____ hours after birth

H.7. If maternal colostrum is hand fed (B or C above), how much colostrum is normally fed during the first 24 hours?

A. Two quarts or less

B. More than 2, but less than 4 quarts

C. Four quarts or more

H.8. Are serum total protein or IgG values routinely measured in calves to assess passive transfer?

Yes_____ No_____

H.9. If YES to previous question, what percentage of calves tested have serum total protein measures ≥ 5 g/dl?

A. > 90% of calves tested

B. 70-89% of calves tested

C. 50-69% of calves tested

D. < 50% of calves tested

H.10. During the past 60 days, what types of **milk or calf starter** have **usually** been fed to preweaned heifer calves **that are kept up to weaning**, after they have received colostrum? Do not include diets that are not fed as a usual practice (e.g., if waste milk is always fed to calves whenever available, mark "yes" for "B," regardless of the number of times it was fed in the past two months. On the other hand, if waste milk was discarded more often than it was fed, mark "no" for "B."

	Included in diet?	If A or B is YES, Is the milk pasteurized?
A. Whole milk from untreated* cows	☐ Yes ☐ No	☐ Yes ☐ No
B. Whole milk from treated* cows (waste milk)	☐ Yes ☐ No	☐ Yes ☐ No
C. Milk replacer without antibiotics	☐ Yes ☐ No	
D. Milk replacer containing antibiotics	☐ Yes ☐ No	
E. Calf starter without antibiotics	☐ Yes ☐ No	
F. Calf starter containing antibiotics	☐ Yes ☐ No	

* "Treated cows" refers to cows that have been given antibiotics and are still within the milk withholding period. (A cow given Naxcel/Excenel is **not** considered a "treated cow" here).

H.11. Are preweaned (milk-fed) calves fed milk ….(circle one)

A. On an individual basis (e.g. individual bucket in hutch or calf pen)
B. In group feeding system (e.g. common trough, automated calf milk feeder)

H.12. Are preweaned (milk-fed) calves fed calf starter … (circle one)

A. an individual basis (e.g. individual bucket in hutch or calf pen)
B. In group feeding system (e.g. common trough, automated calf milk feeder)

H.13. Which of the following coccidiostats or ionophores do you normally use for the following groups of animals? Include products used in feed, water, milk replacer, bolus, or any other form. (Check all that apply)

	Preweaned (milk-fed) calves	Weaned calves up to breeding	Heifers after breeding

Deccox (or other decoquinate product)			
Rumensin (or other monensin product)			
Bovatec (or other lasalocid product)			
Corid (or other amprolium product)			
Sulfaquinoxaline (many oral products)			
Other (Please specify: _____			

H.14. Are probiotics (or direct fed microbials, DFM) fed in the milk diet or starter pellet?
Yes (Specify product name):_____ No_____

H.15. At what age are heifers first introduced to water? _____days old

H.16. At what age are heifers first introduced to starter pellet? _____days old

H.17. At what age are heifers first introduced to forages (e.g. dry hay)? _____weeks old

H.18. At what age are most calves weaned (no longer fed milk)? _____ weeks old

H.19. At what age are most calves grouped for the first time? _____ weeks old

H.20. At what age are most calves dehorned? _____ weeks old

H.21. Which of the following best represents your normal practice regarding the cleaning of calf **milk** buckets or containers between feedings? (Circle one)

1. Between feedings, all calf milk buckets or bottles **washed with water only**.
2. Between feedings, all calf milk buckets or bottles **washed with water and soap**.
3. Between feedings, all calf milk buckets or bottles are **washed and disinfected.** List disinfectant;_____
4. Buckets or containers are not washed or disinfected between feedings on a routine basis.

H.22. In **SUMMER** months are individual calf pens or hutches washed and/or disinfected on
a regular basis after calves are weaned and moved out? (Circle one)

1. Washed with water only. _____times per year
2. Washed with water and soap only. _____times per year
3. Washed and disinfected. _____times per year
   List disinfectant:_____
4. Not washed or disinfected.
5. Calf pen or hutch is not used.

H.23. In **WINTER** months are individual calf pens or hutches washed and/or disinfected on a regular basis? (Circle one)

1. Washed with water only. _____times per year
2. Washed with water and soap only. _____times per year
3. Washed and disinfected. _____times per year
   List disinfectant:_____
4. Not washed or disinfected.
5. Calf pen or hutch is not used.

H.24. How often are individual hutches moved to a new location? (Choose one)

1. Every time a calf is weaned. (Before introducing each new calf.)
2. Not after every weaning, but on a regular basis: → _____times per year
3. Calf hutches are not relocated.
D. Calf hutches are not used.

H.25. Do personnel on your farm use any of the following precautionary practices when handling calves? (Check all that apply)

	After handling each calf	When finished with all calves (e.g., before entering a different area of farm)	Do not routinely use this practice when handling calves
A. Wash boots or use boot dip			
B. Wash hands or use disposable gloves			

I. HEALTH MANAGEMENT AND ANTIBIOTIC USE

I.1. Does this operation routinely record treatments for the following groups of cattle?

	Treatment recorded?	If YES, what types of records are kept? (Check all that apply)			
		Computer	Barn sheet, or notebook	Calendar	Other (specify)
A. Lactating cows	☐ Yes ☐ No				
B. Non-lactating cows	☐ Yes ☐ No				
C. Calves and heifers	☐ Yes ☐ No				

I.2. Where do you get recommendations on the following aspects of antibiotic use? (Check all that apply)

	Veterinarian	Pharmaceutical Representative	Personal Experience	Product label (Manufacturer label)	Other farmers	Other (Please specify)
Recommended use (i.e., what drugs to use for certain diseases)						
Dosage						
Withdrawal Time						

I.3. In the past 2 months report the number of animals that received at least one antibiotic treatment (intramammary, injection or oral):

Problem	Cows (milking or dry)		Bred Heifers		Heifer Calves (preweaned or weaned)	
	# treated	# died	# treated	# died	# treated	# died
Clinical Mastitis						
Uterine Infections						
Foot						

Problems						
Respiratory Disease						
Scours						

I.4. What percentage of animals that die on the farm are necropsied (posted) by the veterinarian?

A. Adult cows: _____%
B. Calves/heifers: _____%

I.5. List the vaccines used in **preweaned heifers, postweaned heifers and bred heifers**, and time of administration

	Product Name	Age at Administration*
A.	_____	_____
B.	_____	_____
C.	_____	_____
D.	_____	_____
E.	_____	_____
F.	_____	_____
G.	_____	_____
H.	_____	_____
I.	_____	_____

* Indicate when vaccine given. E.g. 10 days old, 8 weeks old, 12 months old

I.6. List the vaccines used in **adult cows (dry and milking cows)**, and time of administration

	Product Name	Time of Administration*
A.	_____	_____
B.	_____	_____
C.	_____	_____

D. _____ _____

E. _____ _____

F. _____ _____

G. _____ _____

H. _____ _____

I. _____ _____

* Indicate when vaccine given. E.g. 10 days in milk, at dry off, 3 weeks pre-calving

I.7. Which person in your operation identifies (I), decides (D), and performs (P) **clinical mastitis treatments**?

	I (Identifies)	D (Decides)	P (Performs)
Owner / herd manager			
Milking manager			
Milker			
Veterinarian			
Other (specify:_____)			

I.8. How do you **identify** clinical mastitis? (circle all that apply)

A. Observe for abnormal milk every milking
B. Observe for abnormal milk once per day (or less often, specify: _____)
C. Abnormal milk on milk filter
D. CMT positive
E. Swollen quarter
F. Decrease milk yield & sick cow
G. Other ……
A. I don't know

I.9. Do you routinely **culture** clinical cases in guiding your decision to treat or not treat with antibiotics or to select which antibiotic to use?

A. None
B. Repeat cases

C. Selected cases
D. Only severe cases
E. All cases are cultured

I.10. In the past 2 months, what percent of cows received **intramammary** dry cow therapy that contains antibiotics in **all quarters**? _____%

I.11. In the last 2 months, which **dry cow intramammary antibiotic products** have you used **at dry off**? (select all that apply):

A. Albadry/Novodry
B. Biodry
C. Cefa-Dri
D. First Choice
E. Gally/ErythroDry
F. Go-Dry
G Quartermaster
H Tomorrow
I. Orbenin DC
J. Spectramast DC
K. OrbeSeal
L. "Organic" or "Natural" products
Specify:
_____

I.12. For the last 2 months, which **lactating cow intramammary antibiotic products** have you used to treat **clinical mastitis in lactating cows**? (select all that apply):

A. Aquamast
B. Cefa-Lak
C. Dariclox
D. Gallimycin 35
_____
E. Masti-Clear
F. Pirsue
G. ToDAY
H. Spectramast LC
I. Organic or natural products:
J. Other:
_____

I.13. In the last 2 months, list any **systemic products** have you used to treat clinically ill adult cows (check all that apply)?

Product Name	**Disease Condition – Lactating or Dry Cows**			
	Clinical Mastitis	Uterine Infections	Respiratory Disease	Lameness
Ceftiofur (e.g. exceed, excenel, naxcel)				

Tetracycline (e.g. Liquamycin LA, Bio-mycin 200, Agrimycin)				
Penicillin				
Ampicillin (e.g. Polyflex)				
Erythromycin (e.g. Gallymycin)				
Nuflor				
Tylan 200				
Tylsin				
Micotil				
Draxxin				
Antiinflammatory products (e.g. Aspirin, Banamine)				
Organic or natural products (specify)				
Other (specify)				

I.14. Do you routinely use **footbaths** to control or treat lameness?
Yes _____
No_____

A. **If YES**, do you routinely use antibiotics in footbaths Summer months Yes _____ No_____
Winter months Yes_____ No _____

B. Please list what antibiotics are used, if any:_____

CALVES / HEIFERS

I.15. If antibiotics ARE fed in milk replacer or starter or grower pellet, list the types of antibiotics used below. If unknown, ask to look at tag of bag/container. List only antibiotics here (do not include coccidiostats or ionophores).

A. Antibiotics in milk replacer: _____
B. Antibiotics in calf starter pellet fed preweaning: _____
C. Antibiotics in calf grower pellet fed postweaning: _____

I.16. Do you routinely **use any medications in feed or water in weaned calves or heifers** (other than coccidiostats)? ..... Yes____ No____

A. **If YES**, do you use the additives on a continuous basis?..... Yes____ No_____

B. Please list what feed or water additives are used, if

any_____

I.17. Do you routinely give all heifers (even if not sick) an injection of antibiotics at a scheduled age or time (example. All heifers given shot of long acting antibiotic at weaning)?

Yes_____

No_____

A. **If Yes**, please name systemic product injected: _____

B. **If Yes**, at what age is this shot given? _____ weeks old

I.18. In the last 2 months, list any **systemic products** have you used to treat clinically ill

calves or heifers (check all that apply)?

Product Name	Disease Condition – Calves or Heifers			
	Scours	Respiratory Disease	Joint / umbilical infections	Other
Ceftiofur (e.g. exceed, excenel, naxcel)				
Tetracycline (e.g. Liquamycin LA, Bio-mycin 200, Agrimycin)				
Penicillin				
Ampicillin (e.g. Polyflex)				
Erythromycin (e.g. Gallymycin)				
Nuflor				
Tylan 200				
Tylsin				
Micotil				
Draxxin				
Fluids/electrolytes (oral or injectable)				
Antiinflammatory products (e.g. Aspirin, Banamine)				
Organic or natural products (specify)				
Other (specify)				

University of Minnesota Dairy Health Surveillance Project

Fall, 2009 Survey: MANAGEMENT CHANGES?

Farm:

Instructions: This is a VERY short (1-page) questionnaire asking if you have changed any important management strategies since you filled out the large questionnaire earlier this summer. Please complete the survey and store in the freezer with the bulk tank samples for pick up by the study technician. Thank you!

1. Are new drugs being used on the farm since completing the big questionnaire? **Yes____ No ____**

If yes, please list new drugs:

New Drug Name	Approx. Month Started Using Drug	Disease(s) using new drug for (e.g. mastitis, calf scours)
_____:	_____:	_____
_____:	_____:	_____
_____:	_____:	_____

2. Are there any drugs that you have stopped using since completing the questionnaire? **Yes___ No ___**

If yes, please list old drugs that you have discontinued using:

Old Drug Name	Approx. Month Stopped Using Drug	Disease(s) used old drug for (e.g. mastitis, calf scours)
_____:	_____:	_____
_____:	_____:	_____
_____:	_____:	_____

3. Has your milk diet changed that you feed to the calves < 2 months old? **Yes____ No change ____**

If yes, describe the new milk diet: ____ a) Waste milk - raw
____ b) Waste milk - pasteurized
____ c) Milk replacer with antibiotics
____ d) Milk replacer without antibiotics

4. Has your grain diet changed that you feed to calves < 4 months old? **Yes____ No change ____**

If yes, describe the new grain diet: _____ a) Contains antibiotics. Drug name:_____
_____ b) Does not contain antibiotics

5. Have you purchased any new animals into the herd since summer, 2009?

_____ No, I have not purchased any new animals
_____ Yes, I purchased adult cows or springing heifers (> 18 months old)
_____ Yes, I purchased youngstock (4 to 18 months old)
_____ Yes, I purchased calves (0 to 4 months old)

Chapter 5
Concluding Remarks

This is the first report of comprehensive characterization of MRSA on dairy farms in Minnesota. We report a low prevalence of 1.3% in BTM and 4% in dairy farms. Multidrug resistant strains among the MSSA isolates constituted 17% of the total MSSA isolates indicating moderate proportions of multiple drug resistance among MSSA isolates. This scenario of methicillin and multiple antibiotic resistance although not alarming, merits further monitoring to prevent the emergence and explosive dissemination of clonal types similar to LA MRSA.

The current prevalence of MRSA in dairies is surprising given the large-scale use of antibiotics in dairies over several decades. Antibiotic resistance to methicillin emerged rapidly in hospitals due to the intensive use of antibiotics selecting for resistant microorganisms. One explanation for the low numbers detected could be that current culturing methods do not adequately capture the holistic picture of bacterial fauna in dairy herds. However, similar results of low prevalence were obtained in the NAHMS 2007 study which used PCR to detect MRSA in BTM samples (99).

Another possible explanation for their low prevalence could be because these isolates may not have yet acquired host specific adaptations for survival in dairy farms and hence account for their small numbers. However, given the widespread emergence of a clonal MRSA strain in pigs in the past decade, it is essential to monitor antibiotic resistant pathogens on dairy farms.

The use of different classes of antibiotics for treatment of several diseases on farm

did not show any significant associations with the occurrence of MRSA or MDR MSSA. However, trends ($P<0.15$) were observed for the use of phenicols and tetracyclines and the occurrence of MDR MSSA. Limited time periods and resistance events may account for the failure to detect any significant associations between patterns of antibiotic use and emergence of resistant organisms.

The presence of MRSA on dairies also poses a potential public health risk to farm workers and dairy personnel who are in contact with the cows, milking equipment or raw milk. Although *S. aureus* is killed by pasteurization, the production of heat stable enterotoxins capable of retaining immunologic activity after pasteurization is a cause for concern. Further research needs to be carried out to identify areas of entry and spread of MRSA in dairies and current milking methods and practices may need to be reviewed to implement preventive methods for spread of MRSA within dairies.

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