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Association between Antimicrobial Use and Antimicrobial Resistance in Bovine Mastitis Pathogens

by

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ABSTRACT

Concurrent data on antimicrobial use (AMU) and resistance in food-animal production systems are required to formulate policies for containing antimicrobial resistance (AMR) in bacteria. The specific objectives of this PhD study were four-fold:

1) Determine AMU on Canadian dairy farms,
2) Determine AMR profiles of common bovine mastitis pathogens *Staphylococcus aureus, Escherichia coli,* and *Klebsiella* species isolated from intramammary infections and (sub) clinical mastitis cases,
3) Predict diagnostic accuracy and agreement of Sensititre® MIC susceptibility system and agar disk diffusion test method employed for AMR profiling of udder pathogens, and
4) Determine herd-level association between AMU and AMR in bovine mastitis pathogens.

The data originated from 89 dairy farms located in four regions of Canada. Producers and farm personnel were asked to deposit empty antimicrobial containers in specially provided receptacles to quantify AMU. Three sets of milk samples were collected in the winter and summer of 2007 and 2008. Minimum inhibitory concentrations were determined using Sensititre® bovine mastitis plate and NARMS Gram-negative penal.

Sensititre® and the agar disk diffusion test methods had a moderate to high diagnostic accuracy, and very good essential and categorical agreement for most udder pathogen-antimicrobial combinations. ß-lactams, penicillin combinations, tetracyclines,

trimethoprim-sulfonamide combinations and lincosamides were most commonly used. Prevalence of AMR in *S. aureus*, *E. coli* and *Klebsiella* species isolates was 20.4, 17.7 and 36.6%, respectively. Resistance to penicillin and tetracycline was most common among Gram-positive and Gram-negative isolates, respectively.

Use of penicillin-novobiocin combination administered for dry cow therapy, and systemically administered penicillin was positively associated with penicillin resistance in *S. aureus* isolates; use of pirlimycin for clinical mastitis treatment was associated with pirlimycin resistance. Systemic administration of tetracycline and penicillin was associated with tetracycline resistance in *E. coli*, while ampicillin resistance in *E. coli* was associated with systemic administration of ceftiofur and penicillin. Tetracycline use and resistance were associated in *Klebsiella* species isolates.

In conclusion, ß-lactams were most commonly used for mastitis treatment and control. Prevalence of resistance in bovine mastitis pathogens was low. Herd-level use of certain antimicrobials was associated with AMR in udder pathogens. Sensititre® and the agar disk diffusion method can be readily employed in veterinary diagnostic laboratories.

PREFACE

The following manuscripts have been published or accepted for publication. Vineet Saini obtained and analyzed the data, interpreted the results, and wrote the papers with guidance from his thesis committee (Drs. Herman Barkema, J McClure, David Léger, Patrick Boerlin and Douglas Morck). All authors contributed important intellectual content and provided critical review of the papers. Written permission for reproduction of the article in its entirety has been obtained from the publisher.

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DEDICATION

This thesis is dedicated to my wife, Palvinder, whose unwavering support – emotional and motivational – has led me to this day. The glowing spirits of my belated revered parents, Piara Singh Saini and Krishna Saini helped foster my stalwart resolve to see my PhD through to its fruition.

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LIST OF SYMBOLS, ABBREVIATIONS, NOMENCLATURE

Symbol	Definition
ADD	Agar Disk Diffusion (Chapter1, 2)
ADD	Animal Defined-Daily Dose (Chapter 3)
ADUR	Antimicrobial Drug Use Rate
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
AMXCLA	Amoxycillin – Clavulanic acid Combination
AUC	Area under Curve
BW	Body Weight
CBMRN	Canadian Bovine Mastitis Research Network
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase-Negative Staphylococci
CVP	Compendium of Veterinary Products
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Program
DHI	Dairy Herd Improvement
ESBL	Extended-Spectrum Beta-Lactamase
FINRES-VET	Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents
IMI	Intramammary Infection
IU	International Unit

LMHBCM	Mueller-Hinton Broth with 5% Laked Horse Blood
LOA	Limits of Agreement
MARAN	Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands
MDR	Multi-Drug Resistance
MHB	Mueller-Hinton Broth
MHBCM	Mueller-Hinton Broth with Ca^{2+} and Mg^{2+}
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NARMS	National Antimicrobial Resistance Monitoring System
NMC	National Mastitis Council
OR	Odds Ratio
PCR	Polymerase Chain Reaction
QC	Quality Control
R	Ratio
ROC	Receiver-Operating Characteristic
SCC	Somatic Cell Count
TMP	Trimethoprim
TMPS	Trimethoprim – Sulfamethoxazole Combination

EPIGRAPH

If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

Chapter One: General Introduction

1.1 Mastitis in Dairy Cattle

Bovine mastitis is caused by entry of bacteria in the mammary gland leading to inflammation (Bramley et al., 1996). This dynamic disease, in which "infection and inflammation wax and wane" (Sandholm et al., 1990) is marked by physical and chemical changes in the milk, and pathological changes in the glandular tissue (Radostits et al., 2000). Bovine mastitis is generally classified into clinical and subclinical mastitis. Clinical mastitis is characterized by local (e.g. swelling of the udder, heat and pain) or systemic (e.g. fever, anorexia, depression) symptoms with milk abnormalities (e.g. milk clots, flakes, watery secretions, blood), where as subclinical mastitis is marked by high SCC, milk production losses and lowered milk quality (Gruet et al., 2001). Bovine mastitis remains the most common, most frequently treated, and most costly infectious disease of dairy cattle (Kossaibati, 1997). It is also the number one reason for use of antimicrobials on dairy farms (Mitchell et al. 1998).

Of the 135 infectious agents associated with clinical mastitis episodes in dairy cattle, the most commonly isolated are *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae,* and *Escherichia coli* (Bramley et al., 1996; Watts, 1988). *S. aureus* and *E. coli* are the most frequent causes of contagious and environmental clinical mastitis in dairy cattle, respectively (Barkema et al., 1998; Olde Riekerink et al., 2008), while *Klebsiella* is the most frequently found clinical mastitis pathogen in free-stall dairy cattle herds in Western Canada (Olde Riekerink et al., 2008). *S. aureus* remains one of the most important causes of contagious clinical mastitis, and the most frequently

isolated pathogen in subclinical mastitis cases worldwide (Waage, 1997; Barkema et al., 1998; Roberson et al., 1998; Sargeant et al., 1998; Waage et al., 1999; Olde Riekerink et al., 2008; Sampimon et al., 2009). Its ubiquitous presence in dairy herds is potentially due to its ability to cause chronically recurring infections, and to its resistance to antimicrobial treatment (Wilson et al., 1999). Coliforms cause environmental mastitis in dairy cattle mostly in early lactation with local and more often severe systemic signs than Gram-positive mastitis (Barkema et al., 1998). The majority of these coliforms are *E. coli* that originate from the cow's environment and infect udder via the teat canal (Kaipainen et al., 2002; Lehtolainen et al., 2003). These Gram-negative udder pathogens have been implicated in as low as 20% to more than 60% of clinical mastitis cases in different countries (Pyörälä and Honkanen-Buzalski, 1994; Shpigel et al., 1998). Despite the advances in the control of bovine mastitis, current levels of disease caused by the udder pathogens remain a persistent problem (Sargeant et al. 1998; Leigh, 1999; Waage et al., 1999).

1.2 Treatment of Bovine Mastitis

Antimicrobial therapy is the preferred approach for treating bovine mastitis cases (Radostits et al., 2000; Erskine et al., 2002). For an antimicrobial to be effective, it must reach and persist at the site of infection in effective concentration. Antimicrobials are either administered intramammarily or systemically for decreasing the incidence and duration of udder infections in dairy cattle. Systemic antimicrobial therapy is preferred in cases of bacteremia potentially due to coliform mastitis (Wagner and Erskine, 2006) or when the udder is swollen thereby indicating that the milk duct system is swollen, compressed or blocked by inflammatory debris and that the site of infection is inaccessible to an

antimicrobial agent (Gruet et al., 2001). However, the rate of passage of an antimicrobial into milk after parentral administration depends upon degree of ionization, lipid solubility, and extent of plasma-protein binding (Baggot, 2006). In general, only the lipid-soluble, non-ionized and plasma protein unbound fraction of an antimicrobial can penetrate the blood-milk barrier to enter into milk and diffuse into transcellullar fluid. On the contrary, the intramammary route of administration has the potential to provide higher and persistent drug concentration than systemic administration (Walker and Giguère, 2006), thereby enabling smaller amounts of an antimicrobial to be used.

Antimicrobials are commonly administered for appropriate management of clinical mastitis during lactation and effective dry cow management as a part of ten points recommended by National Mastitis Council (NMC)'s mastitis control program. Dry cow therapy is intended to cure existing infections and prevent new infections during the dry period (Gruet et al., 2001; Aarestrup, 2004). Treatment of intramammary infection (IMI) at dry off has many advantages over treatment during the lactation such as higher dosage of an antimicrobial can be administered safely, a more uniform level of antimicrobial is maintained over longer duration, higher cure rate, lower risk of contamination of milk with antibiotic residues, and no discard of milk; incidence of new IMI during non-lactating period and clinical mastitis at freshening is also reduced (Nickerson, 1991). However, the antimicrobials persist during the early and mid dry period and not throughout the entire duration of the average 60 day dry period (Gruet et al., 2001). The use of antimicrobials during lactation and non-lactating period is hypothesized to select for antimicrobial resistance (AMR) in bovine mastitis pathogens.

A limited number of antimicrobial drug classes are indicated for intramammary treatment and prevention of bovine mastitis. Antimicrobial classes as cephalosporins (cephapirin sodium, cephapirin benzathine, ceftiofur hydrochloride), penicillins (cloxacillin benzathine, penicillin G procaine – novobiocin combination), penicillin combinations (penicillin G procaine – dihydrostreptomycin sulfate – novobiocin sodium – polymyxin B sulfate combination), macrolides (erythromycin), and lincosamides (pirlimycin) are most commonly administered intramammarily either during the lactation or dry period for treatment and (or) prevention of Gram-positive mastitis in dairy cattle (Pol and Ruegg, 2007). Antimicrobials such as oxytetracycline, sulfadimethoxine, ceftiofur, ampicillin and amoxicillin have an appropriate spectrum of activity against *E. coli* and *Klebsiella* species isolates (Wagner and Erskine, 2006). In general, broad-spectrum antimicrobials are commonly used to treat coliform mastitis (Erskine et al., 2002); although there is no convincing evidence of the efficacy (Erskine et al., 1992; Myllys et al., 1998; Pyörälä et al., 1994). Similarly, despite best possible antimicrobial treatments, bacteriological cure failures are common in *S. aureus* mastitis. Antimicrobial resistance is considered as one of the reasons for low cure rates in bovine mastitis pathogens (Barkema et al., 2006).

1.3 Antimicrobial Resistance in Bovine Mastitis Pathogens

There are many definitions of AMR depending upon the criteria for classification such as genetic, biochemical, microbiological and clinical (Guardabassi and Courvalin, 2006). Microbiological and clinical AMR definitions are most commonly used. A strain is classified as resistant according to microbiological definition, when it grows in the presence of higher concentration of an antimicrobial than other strains of the same species. Clinical definition of AMR is defined as the ability of a microorganism to withstand the effect of a

normally acquired concentration of an antimicrobial at the site of infection following standard treatment procedures (Witte, 1998).

The emergence of AMR is not an unexpected phenomenon. In fact, antimicrobials and AMR have closely followed each other since the origin of antimicrobials (Davies, 1997). Even before the commercial production and use of antimicrobials in human and veterinary medicine, antimicrobial resistant bacteria existed (McDermott et al., 2002). Presumably, the emergence of AMR in the antibiotic-producing bacteria was a mechanism to protect them from their own produce (Dancer, Shears, and Platt 1997). The commercial production and use of antimicrobials after late 1940s in animal agriculture resulted in effective treatment of infections, previously thought to be untreatable. Those developments were harbingers of the "wonder drug era" (Prescott, 2006). However, soon after, AMR began emerging and rising in the bacteria of human and animal origin at alarming rate (Kammer, 1982). Concerns are also rising about the transfer of AMR determinants from animal to human populations along the food chain (White and McDermott, 2001). Reduced efficacy of treatment, increased morbidity, mortality, and health-care costs are considered as the aftermath of AMR in bacterial pathogens (Travers and Barza, 2002; Witte, 1998). Various national and international bodies have therefore recommended coordinated ongoing surveillance of AMR in pathogens and potential pathogens in human and veterinary medicine (Nicholls et al., 2001; WHO, 2001).

In Canada, Sabour et al. (2004) conducted a study to determine AMR in 288 *S. aureus* isolates from clinical mastitis cases on 58 Eastern Canadian dairy farms in three provinces (Ontario, Québec, Prince Edward Island). Twenty five percent of isolates were resistant to

one or more antimicrobials tested (penicillin, pirlimycin, tetracycline, ceftiofur, tilmicosin, erythromycin, penicillin-novobiocin combination, cephalothin, oxacillin, and sulfadimethoxine). Resistance to penicillin (10.0%) was most common followed by resistance to sulfadimethoxine (8.0%). Multi-drug resistance was rare. Geographical variation in the prevalence of AMR was observed; isolates from Ontario exhibited the highest prevalence (30.0%), followed by Québec (20.0%) and Prince Edward Island (19.0%). No isolate was found resistant to penicillin-novobiocin combination, and cephalosporins (ceftiofur, cephalothin). In case of bovine mastitis coliforms, resistance proportions in *E. coli* isolates ranged from 5.0 to 37.0% for tetracycline (FINRES-Vet, 2007; Makovec and Ruegg, 2003), 7.0 to 34.0% for sulfisoxazole (FINRES-Vet, 2007; Srinivasan et al., 2007), 0 to 5.0% for ceftiofur (FINRES-Vet, 2007; Erskine et al., 2002), and 7.0 to 21.0% for ampicillin (Lanz et al., 2003; Lehtolainen et al., 2003) across various studies worldwide. Resistance proportions in *Klebsiella* species isolates also varied greatly from 7.0 to 33.0% for tetracycline (Bengtsson et al., 2009; Erskine et al., 2002), 10.0 to 12.0% for sulfisoxazole / sulfamethoxazole (Bengtsson et al., 2009; Makovec and Ruegg, 2003) and 0 to 14.0% for ceftiofur (FINRES-Vet, 2007; Erskine et al., 2002) across studies. Multi-drug resistance was common in bovine mastitis coliforms. In general, resistance to various antimicrobials is frequently seen in bovine mastitis isolates (Güler et al., 2010; Watts and Salmon, 1997; Makovec and Ruegg, 2003).

1.4 Association between Antimicrobial Use and Resistance in Bovine Mastitis Pathogens

Increasing prevalence of AMR, and the associated negative health outcomes have lead to intense scrutinization of the factors promoting the emergence and dissemination of

resistance among pathogens (and potential pathogens) in humans and animals (Bager et al., 1999; Codex 2005; WHO 2000). Antimicrobial use (AMU) in human and veterinary medicine is considered to be the main driver for emergence of resistance in bacteria (Levy and Marshall, 2004). The increased prevalence and dissemination of AMR is in line with the Darwinian principal of "survival of the fittest" (Boerlin and White, 2006). Antimicrobial use over longer duration changes the microbial ecology in a given environment such that resistant strains become dominant in the bacterial population (Levy, 1998).

Variation in antimicrobial susceptibility among bacteria of the same species from different sites of infection in different animal species is observed. For example, Lanz et al. (2002) observed differences in resistance frequency of clinical *E. coli* isolated from different disease processes in pigs, dairy cattle, dogs and cats, and laying hens in Switzerland. It would be preposterous to assume that factors impacting AMR frequency in bacteria isolated from different sites of infection or from different animal species might be similar. Various pharmacokinetic and pharmacodynamic factors influence the therapeutic effect of an antimicrobial agent and potentially AMR in the invading pathogen. For example, therapeutic effects of an antimicrobial agent depend upon the site and nature of the infectious disease process (Martinez et al., 2006). Therefore, results from studies describing risk factors of AMR in bacteria in one animal species or from one site of infection might not be extrapolated to a different animal species or a different site of infection.

There is a lack of studies describing relationship between AMU and AMR in bovine mastitis pathogens. Few studies have determined AMR in bovine mastitis pathogens

isolated from dairy farms with varying levels of AMU exposure (Pol and Ruegg, 2007; Rajala-Schultz et al., 2004; Roesch et al., 2006; Tikofsky et al., 2003); however, the results have been conflicting. None of these studies modeled AMR in bovine mastitis pathogens by including AMU and non-AMU factors (e.g. managemental practices) that could potentially impact AMU-AMR association. In case of coliforms, there are various studies describing association between AMU and AMR in coliforms isolated from the feces of young dairy calves, dairy cattle, beef cattle and swine (Akwar et al., 2008; Berge et al., 2005; Berge et al., 2010; Checkley et al., 2008). However, studies describing the impact of therapeutic and prophylactic AMU on AMR in bovine mastitis *E. coli* and *Klebsiella* species isolates are rare (Srinivasan et al., 2007). Due to paucity of such studies, there is a lack of convincing evidence that AMU for mastitis treatment and control is associated with AMR in bovine mastitis pathogens in a dairy farm environment (Hillerton and Berry, 2005).

1.5 Antimicrobial Susceptibility Testing of Bovine Mastitis Pathogens

Antimicrobial susceptibility testing of udder pathogens is an important step in defining appropriate farm-level treatment protocols. Determining accuracy and precision of a measuring instrument is therefore of paramount importance in antimicrobial susceptibility testing. Phenotypic and genotypic methods of antimicrobial susceptibility testing and detection of resistance are being used commonly in veterinary diagnostic microbiology. Among phenotypic methods of antimicrobial susceptibility testing, detection of resistance in bacterial isolates is commonly done using agar disk diffusion (ADD) method of Bauer et al. (1966). The ADD method has long been used in veterinary diagnostic microbiology due to simplicity, low cost, and flexibility in type and number of drugs that can be tested

(Walker, 2000). The results are reported qualitatively as sensitive, intermediate or resistant depending upon the zone of inhibition diameter cut-off. On the other hand, dilution methods such as agar, and broth macro/micro dilution yield valuable quantitative information about decreased susceptibility, or emerging resistance in bacterial pathogens in terms of minimum inhibitory concentrations (MIC). Quantitative results in form of MIC relate the qualitative results to time-varying concentrations of antimicrobials at the site of injection (Wertz et al., 1978). In general, dilution methods are usually considered to be the "gold standard" (Walker, 2000). However, methods of antimicrobial susceptibility testing can yield erroneous results under non-standardized testing conditions. It is becoming increasingly important to ascribe to standardized testing procedures such as those advocated by regulatory agencies or professional organizations (e.g. Clinical and Laboratory Standards Institute, CLSI). Therefore, whenever susceptibility testing is to be performed, standardized testing procedures and conditions using accepted guidelines, and appropriate quality assurance should be adhered to (White et al., 2001).

Automated susceptibility testing methods are being increasingly used in veterinary diagnostic microbiology. Sensititre® (TREK Diagnostic Systems, Cleveland, Ohio) is one commercial MIC susceptibility system that is a modification of broth microdilution test method; it is referenced with CLSI standards (Gavan et al., 1980; Doern et al., 1985). Various studies in human medicine have assessed diagnostic agreement of Sensititre® with reference to manual broth microdilution test method for stock organisms and clinical isolates (Gavan et al., 1980; Hansen and Freedy, 1983; Jones et al., 1980). In veterinary medicine, although many diagnostic laboratories are using commercial antimicrobial susceptibility systems, there is a dearth of validation studies data in this regard (Watts and

Yancey, 1994). To date, the Sensititre® has not been compared to a reference manual broth microdilution MIC test method for bovine clinical mastitis pathogens.

1.6 Objectives

The objectives of this PhD study were a) to predict diagnostic accuracy of Sensititre® MIC mastitis panel and the ADD method in reference to a manual broth microdilution test method for determining antimicrobial susceptibility profiles of clinical bovine mastitis pathogens, b) to quantify antimicrobial drug utilization on Canadian dairy farms, c) to determine frequency of antimicrobial resistant bovine mastitis *S. aureus*, *E. coli* and *Klebsiella* species pathogens, and d) to assess and evaluate if a herd-level association exists between AMU and AMR in bovine mastitis pathogens.

1.7 Thesis Organization

This thesis examines relationship between antimicrobial use and antimicrobial resistance in common bovine mastitis pathogens. Each chapter reports on a unique thesis component formatted for independent publication as part of a paper-based thesis, however, all components are linked by the *common objective of improving knowledge of the antimicrobial resistance outcomes in common mastitis pathogens associated with antimicrobial use in dairy cattle.*

Chapter one describes about determining diagnostic accuracy and agreement of Sensititre® MIC bovine mastitis panel and the ADD method in reference to a manual broth microdilution test method. Chapter two describes qualitative and quantitative aspects of antimicrobial drug utilization on Canadian dairy farms. Antibiograms of *S. aureus*, *E. coli*

and *Klebsiella* species isolated from IMI, (sub) clinical bovine mastitis cases are described in chapter three. Chapter four describes a herd-level association between AMU and resistance in *S. aureus* isolates, where as chapter five describes a relationship between AMU and AMR in bovine mastitis coliforms. Finally, overall conclusions and future perspectives form chapter six.

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Chapter Two: Diagnostic accuracy assessment of Sensititre® and agar disk diffusion for determining antimicrobial resistance profiles of bovine clinical mastitis pathogens

2.1 Abstract

Determining accuracy and precision of a measuring instrument is pertinent in antimicrobial susceptibility testing. This study was conducted to predict diagnostic accuracy of Sensititre® MIC mastitis panel (Sensititre®) and agar disk diffusion (ADD) method with reference to manual broth microdilution test method for antimicrobial resistance profiling of *Escherichia coli* (n=156), *Staphylococcus aureus* (n=154), streptococci (n=116) and enterococci (n=31) bovine clinical mastitis isolates. Isolates were tested against ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, penicillin-novobiocin combination, pirlimycin, and tetracycline. Diagnostic accuracy was determined by estimating area under the receiver–operating characteristic curve; inter–test essential and categorical agreement was determined as well.

Sensititre® and the ADD method demonstrated moderate to highly accurate (71–99%), and moderate to perfect (71–100%) predictive accuracy in 74 and 76% of the isolate–antimicrobial MIC combinations, respectively. However, the diagnostic accuracy was low for *S. aureus*–ceftiofur / oxacillin combinations, and other streptococci–ampicillin combinations by either testing method. Essential agreement between Sensititre® automatic MIC readings and manual broth microdilution test method was 87%. Essential agreement between Sensititre® automatic and manual MIC readings methods was 97%. Furthermore, the ADD test method and Sensititre® MIC method exhibited 92 and 91% categorical (sensitive, intermediate, resistant) agreement results respectively, when compared with the

reference method. However, both methods demonstrated lower agreement for *E. coli* – ampicillin / cephalothin combinations than Gram–positive isolates.

In conclusion, Sensititre® and the ADD methods had a moderate to high diagnostic accuracy, and very good essential and categorical agreement for most udder pathogen–antimicrobial combinations and can be readily employed in veterinary diagnostic laboratories.

2.2 Introduction

Antimicrobial therapy is generally the most common way of treating mastitis in dairy cattle (Mitchell et al., 1998). Unfortunately, despite best possible antimicrobial treatments, bacteriological cure rates (e.g. of *Staphylococcus aureus* mastitis) seldom exceed 50%. Antimicrobial resistance (AMR) is potentially one of the reasons for treatment failures (Barkema et al., 2006), hence antimicrobial susceptibility testing of udder pathogens is an important step in defining appropriate farm–level treatment protocols.

The most common method used for AMR profiling of bacterial isolates is the agar disk diffusion (ADD) method of Bauer et al. (1966). The ADD method has long been used in veterinary diagnostic microbiology due to easy use, low cost, inter–laboratory repeatability, and flexibility in type and number of drugs that can be tested (Walker, 2006). This test has extensively been used for ascertaining antibiograms of bovine mastitis pathogens (McDonald et al., 1977; Owens and Watts, 1988). However, the ADD method is sensitive to changes in operator techniques, and zone of inhibition diameters interpretation, and only qualitative results as sensitive, intermediate, and resistant are obtained. Therefore, to relate these qualitative results to time–varying concentrations of antimicrobials at the site of infection, quantitative results in form of minimum inhibitory concentrations (MIC) were needed (Wertz, 1978). In order to speed up the process of MIC determination, various commercial automated MIC susceptibility systems have been developed. One of the commercial *in vitro* broth microdilution method used in veterinary microbiological diagnostics for AMR profiling is the Sensititre® (TREK Diagnostic Systems, Cleveland,

Ohio). Results can be determined using either automated or manual reading system and is referenced with the CLSI standards (Barry, 1976; Doern et al., 1985; Gavan et al., 1980). The Sensititre® MIC testing system is of particular interest compared to other commercial MIC systems because this system offers a MIC panel specifically for bovine mastitis pathogens.

In human medicine, many studies have determined diagnostic agreement between Sensititre® and manual broth microdilution test method with stock organisms and human clinical isolates for assessing intra and inter–laboratory variations in antimicrobial susceptibility testing (Gavan et al., 1980; Hansen and Freedy, 1983; Jones et al., 1980). In veterinary medicine, although many diagnostic laboratories are using commercial antimicrobial susceptibility systems, there is a dearth of validation studies data in this regard (Watts and Yancey, 1994). Papp and Muckle (1991) compared a commercial microdilution MIC system (Sceptor System) with an agar dilution method for veterinary clinical isolates. Inter-test MIC comparisons were done for Gram-positive and Gram-negative isolates. However, common Gram-negative bovine mastitis pathogens as *Escherichia coli* were not tested, and the animal sources of these veterinary clinical isolates were not described as well. Watson et al. (1991) compared a veterinary breakpoint MIC system with ADD method for common veterinary pathogens. In this study, only a single concentration of various antimicrobials was used, and isolates from bovine mastitis samples were not included. Franklin and Wierup (1982) compared the Sensititre® MIC system to agar dilution method for antimicrobial resistance profiling of veterinary pathogens isolated from different animals, however the inter-test MIC comparisons were made on the genus levels and the animal sources of isolates were not identified. To date, the Sensititre® has not

been compared to a reference broth microdilution MIC test method for bovine clinical mastitis pathogens.

Similarly, other studies involving comparison between MIC susceptibility systems and ADD methods for AMR profiling of veterinary pathogens are limited in scope to a few udder pathogens, and a few antimicrobic drugs used for control and treatment of mastitis (Hoblet et al., 1993; Klement et al., 2005; Schlegelova et al., 2001). These studies did not use the commercial Sensititre® system. Furthermore, Sensititre® automatic readings method has not been compared with manual readings method in the studies involving veterinary pathogens.

The objectives of this study were therefore: 1) to predict diagnostic accuracy of Sensititre® MIC mastitis panel and agar disk diffusion method using manual broth microdilution MIC test method as the reference, 2) to assess diagnostic agreement between agar disk diffusion and manual broth microdilution MIC test method, 3) to assess MIC diagnostic agreement between Sensititre® system and manual broth microdilution MIC test method, and 4) to assess agreement between Sensititre® automatic readings and manual readings test methods in determining AMR profiles of clinical bovine mastitis pathogens.

2.3 Materials and Methods

2.3.1 Herd Selection, Sampling and Bacterial Culturing

Milk samples (n=3033) were obtained from quarters of dairy cows with clinical mastitis in 10 provinces across Canada (Olde Riekerink et al., 2008). In short, dairy farmers were contacted through local veterinary practitioners or provincial Canadian Quality Milk

Program to submit producer–diagnosed clinical mastitis milk samples to the Atlantic Veterinary College at Charlottetown, Canada. A total of 1,441 isolates were cultured from these milk samples from 106 dairy farms. Keeping in mind that multiple isolates could be coming from a single farm, and that antimicrobial resistance in isolates could potentially be a herd-level factor, it was decided to keep number of isolates per farm as low as possible for the purpose of statistical independence. Therefore, 457 isolates were selected for comparing the Sensititre® system with the ADD method. These isolates were lyophilized and stored afterwards. Two years later, out of these 457 isolates, a random subset (n=150, @ 25 isolates per mastitis pathogen) was selected for validating Sensititre® system and the ADD method using manual broth microdilution test method as the reference. However, because not all lyophilized samples could be recultured, a total of 119 isolates were tested finally with the manual broth microdilution test method. Bacterial culturing and identification of the milk samples was done as per National Mastitis Council guidelines (Hogan et al., 1999). The following reference strains were included in the study: *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 49619, and *Escherichia coli* ATCC 25922. Isolates of interest in this study e.g. *S. aureus, Streptococcus uberis, Streptococcus dysgalactiae, E. coli,* other streptococci and *Enterococcus* sp. were stored in skim milk in a commercial freezer at -20°C.

2.3.2 Antimicrobials

Sensititre® Standard Susceptibility Mastitis Plate, CMV1AMAF, consisting of 10 antimicrobials in serial two–fold dilutions was used in the study (Trek Diagnostic Systems). This bovine mastitis plate contains the following antimicrobials: ampicillin, ceftiofur,

cephalothin, erythromycin, oxacillin, penicillin, penicillin-novobiocin combination, pirlimycin, sulfadimethoxine, and tetracycline (Table 2.1). Commercial antimicrobial disks of ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, novobiocin, pirlimycin, and tetracycline were used for ADD method (Table 2.1). Since sulfadimethoxine is hardly used for mastitis treatment and control, it was not used for ADD and manual broth microdilution test method.

2.3.3 Agar Disk Diffusion Method

Bacteria were sub–cultured twice using a Columbia agar plate with 5% sheep blood (Oxoid Canada, Nepean, Ontario, Canada). Thereafter, the inocula were prepared for Sensititre® and ADD tests. The ADD test was carried out based on CLSI guidelines. In short, the inoculum was prepared in sterile demineralized water to 0.5 McFarland turbidity standard for estimating cell density. Seeding of the Mueller–Hinton (Oxoid Canada, Nepean, Ontario) plate was done with the broth suspension using a cotton swab. Antimicrobial disks were then placed on the agar plates. Plates were incubated overnight (18–24 h) at 37°C (Bauer et al., 1966; NCCLS, 2003). Zone of inhibition diameters were measured in millimeters.

2.3.4 Sensititre® System MIC Method

Pure culture, grown overnight on a Columbia agar plate with 5% sheep blood was used for making a bacterial suspension in demineralized water for the Sensititre®. This suspension was standardized to 0.5 McFarland turbidity standard and confirmed using the Sensititre® Nephelometer. Subsequently, a 10µL aliquot was transferred using a calibrated loop into a tube of Sensititre® Mueller–Hinton broth that was finally mixed on a vortex for

approximately 10 seconds. A Sensititre® single–use dose head was placed on the Mueller–Hinton broth tube, and the tube was then placed in the Sensititre® AutoInoculater according to manufacturer's specifications. The AutoInoculater delivered 50µL into each well containing serial two–fold dilutions of antimicrobials on the bovine mastitis plate. After inoculation, the panel was covered with an adhesive seal, and incubated overnight. MIC of different antimicrobial–bacterial combinations were determined manually. Afterwards, the same person recorded the automatic readings by using the Sensititre® Auto Reader so as to prevent bias.

2.3.5 Manual Broth Microdilution Test Method

2.3.5.1 Culture and Inoculum Preparation

A computer-driven method of drawing observations randomly without replacement was used for selecting 119 isolates. These randomly selected isolates were streaked onto a Columbia agar plate with 5% sheep blood. All isolates were incubated at 35°C without CO_2, except for streptococci, which were incubated in the presence of CO_2 to obtain sufficient growth. Well–isolated colonies of fresh isolates (18-24 h) were transferred from the agar plate and diluted in 2mL of physiological saline to attain a 0.5 McFarland turbidity standard.

2.3.5.2 Stock Solutions Preparation

Reference powders of ampicillin, cephalothin, erythromycin, oxacillin, penicillin, novobiocin, and tetracycline were obtained commercially from Sigma-Aldrich® (Sigma–Aldrich Canada Ltd., Oakville, Ontario, Canada) to prepare antimicrobial stock solutions. Ceftiofur and pirlimycin powders were obtained directly from the manufacturer (Pfizer

Animal Health, Kirkland, Québec, Canada). All reference powders were stored as recommended by the manufacturers. The stock solutions were prepared to contain drug concentrations at four times the final concentrations of the highest concentration on the MIC panel.

The stock solutions were sterilized by filtering through a membrane filter. All of the stock solutions were dispensed in tubes and stored at -20°C, except for tetracycline which was stored at 4°C. The tubes were removed as needed, and used on the same day. Any unused solution was discarded at the end of day. Antimicrobial drug concentrations encompassed both the QC range and the CLSI breakpoints.

2.3.5.3 Media Preparation

Mueller–Hinton broth (MHB) (Oxoid CM0405) was made following the manufacturer's instructions and was supplemented with Ca^{2+} and Mg^{2+} (MHBCM) after being autoclaved, and chilled to 4°C. For streptococcal isolates, 5% laked horse blood was added to the M–H broth (LMHBCM).

2.3.5.4 Microdilution Tray Preparation and Inoculation

Using a single pipette, 0.1mL of double-strength MHBCM (42 g / L of distilled water) was added to the first row followed by further additions of 0.1mL of single strength MHBCM (21 g / L of distilled water) to the remaining wells in the microdilution tray. Thereafter, 0.1mL of the antimicrobial stock solution was added to the first row, and later transferred to the remaining wells for serial dilutions, so that the final volume stayed at 0.1mL in the

wells. 0.1mL was discarded after last dilution.

After diluting the standardized inoculum to 1:100 ratio with single strength MHBCM, the inoculum was dispensed in the wells within 15 minutes. Each plate included the CLSI positive reference control well, as well as a series of inoculum free dilution wells serving as a negative control. The plates were sealed and incubated for 18–24 h at 35°C. The MICs were determined based upon presence or absence of turbidity in the wells. Two trained laboratory personnel recorded MICs afterwards.

To ascertain final inoculum density and purity, 0.1 mL of the standardized inoculum was dispensed in 10 mL saline solution, and later 0.1 mL were streaked out on a Columbia agar plate with 5% sheep blood. After incubation for 18–24 h, the plates were checked for purity and the colonies were counted.

2.3.6 Statistical Analyses

Manual broth microdilution test method was used as the reference method. Minimum inhibitory concentration values (as determined by Sensititre® automatic and manual readings method, and reference test method) outside the antimicrobial dilution range were defined as off–scale MICs, while the MICs within the dilution ranges were defined as on–scale or finite MICs. The off–scale MIC pairs were assumed to be in agreement for the sake of statistical analysis. Using CLSI guidelines, the isolates were classified as sensitive, intermediate or resistant.

Receiver–operating characteristic (ROC) analysis methodology was used to assess diagnostic accuracy of Sensititre® and ADD method with reference to manual broth microdilution test method (Greiner et al., 2000). Area under the ROC curve (AUC) was used as a summary statistic. Intermediate category isolates were merged with the resistant category isolates to determine the AUC statistic. Based upon the AUC statistic, the diagnostic test can either be non–informative (AUC=0.5), less accurate ($0.5<AUC \leq 0.7$), moderately accurate ($0.7<AUC \leq 0.9$), highly accurate ($0.9<AUC <1$) and perfect test (AUC=1) (Swets, 1988).

Quantitative agreement for MICs was measured in terms of absolute and essential agreement between different test methods (Sensititre® automatic MIC method compared to reference test method; Sensititre® manual readings method compared to automatic readings method), and the test statistic was inter–test MIC ratio (R). Absolute agreement was defined as percentage of inter–test MIC pairs with R=1. R=2 indicated one dilution underestimation, whereas R=0.5 indicated one dilution overestimation by Sensititre® in comparison to the manual broth microdilution test method ($\pm 1\text{-}\log_2$). Since single two–fold dilution was the inherent variability of the MIC dilution systems, inter–test MICs within this tolerance range were considered to be in an essential agreement. In other words, essential agreement was defined as the percentage of inter–test MIC pairs with values of R=0.5, 1, and 2. Errors were defined as inter–test MIC pairs with values of $R<0.5$ or $R >2$.

Limits of agreement (LOA) analysis was used to assess agreement between Sensititre® automatic MIC readings and manual MIC readings method as well (Bland and Altman,

1986). Limits of agreement values precisely quantified the differences between test methods by comparing the differences in log MIC values determined from respective test methods with the mean of the log MICs.

Proportion agreement analysis method was used to ascertain categorical agreement (sensitive, intermediate and resistant) of Sensititre® automatic MIC and ADD method with the reference manual broth microdilution method. Categorical agreement was defined as percentage accordance between qualitative AMR profile results obtained using Sensititre® automatic MIC or ADD method and the reference method. Very major error, major error, and discrepancy percentages were calculated for different isolate–drug combinations. Very major error was defined as an error in an AMR profile result if an isolate was categorized as resistant by reference test method and sensitive by Sensititre® or ADD method (false–sensitive). Major error was defined as an error in an AMR profile result if an isolate was categorized as sensitive by reference test method and resistant by Sensititre® or ADD method (false–resistant). Discrepancy was defined as an error in an AMR profile result if an intermediate isolate was categorized as sensitive or resistant and vice–versa. Some of the antimicrobial–isolate combinations were not analyzed for categorical agreement due to clinical inappropriateness and /or intrinsic resistance (oxacillin / Penicillin / erythromycin / Pirlimycin–*E. coli*), and lack of interpretive criteria (oxacillin–enterococci / streptococci, tetracycline–enterococci, pirlimycin / ceftiofur–enterococci).

Data analyses were performed using Intercooled Stata™10.1 (Intercooled Stata for Macintosh, version 10.1, Stata Corporation, College Station, TX).

2.4 Results

2.4.1 Sensititre® Automatic MIC Readings Method Compared with Manual Broth Microdilution Test

The AUC estimates ranged from 0.27 to 1.00 (Table 2.2). Sensititre® exhibited the lowest predictive accuracy for *S. aureus*–ceftiofur combination. The predictive accuracy was less than 0.5 in 10% of the isolate–antimicrobial combinations. Overall, Sensititre® was non–informative (AUC=0.5), less accurate (0.5<AUC≤0.7), moderately accurate (0.7<AUC≤0.9), highly accurate (0.9<AUC<1) and perfect (AUC=1) in its predictive accuracy in 7, 0, 53, 21, and 9% of the isolate–antimicrobial combinations compared to the reference manual broth microdilution MIC method, respectively.

Absolute agreement (R=1) between respective MIC values (off and on–scale) was evident in 12 to 100% of various isolate–antimicrobial combinations, the lowest in *E. coli*–cephalothin combination (Table 2.3). Essential agreement between test methods was evident in 16 to 100% of the isolate-antimicrobial combinations, the lowest for *E. coli*–tetracycline combination. Among various isolate–antimicrobial combinations, the underestimation bias was evident in 0 to 84% of isolate–antimicrobial combinations, the highest in *E. coli*–tetracycline combination. Overestimation bias was evident in 0 to 15% of different isolate–antimicrobial combinations. Overall, Sensititre® exhibited underestimation and overestimation bias in 11 and 2% of the isolate–antimicrobial combinations; absolute and essential agreement was evident in 75 and 87% of the isolate–antimicrobial combinations, respectively.

Overall essential agreement values across Gram–positive isolates ranged from 79 to 95%, the lowest value was evident for enterococcal isolates (Table 2.4). Overall essential agreement value for all Gram–positive isolates was 89%. Among Gram–positive isolates, essential agreement across different antimicrobials was the highest among other streptococci (95%), followed by *S. aureus* (93%), *Strep. uberis* (89%), *Strep. dysgalactiae* (88%), and enterococci (79%). *Escherichia coli*–different antimicrobials combinations had a far lower overall essential agreement value (79%).

Overall categorical agreement was 91%; very major errors, major errors, and discrepancies being 3, 1, and 5% respectively. Categorical agreement between test methods ranged from 32 to 100% for specific isolate–antimicrobial combinations (Table 2.5). Inter–test categorical agreement was the lowest for *E. coli*–cephalothin combination with very major errors and discrepancies occurring 20 and 48% of the time, respectively. Furthermore, *E. coli*–ampicillin, and *Strep. dysgalactiae*–tetracycline combinations had low categorical agreement values (72 and 50%). *Escherichia coli*–tetracycline, *Strep. uberis*–tetracycline / pirlimycin, and *Strep. dysgalactiae*–tetracycline / pirlimycin combinations had notably higher values of very major errors (≥ 10%).

2.4.2 Sensititre® Manual MIC Readings Method Compared with Automated Readings Method

Sensititre® manual MIC readings method exhibited absolute and essential agreement with automated readings method in 94 and 97% of the isolate–antimicrobial combinations, respectively. Absolute and essential agreement between respective MIC values (off and on–

scale) was evident in 76 to 100%, and 83 to 100% of the various isolate–antimicrobial combinations. Notably, ≥ 10% errors were evident in *Strep. uberis*–pirlimycin (17%), *S. aureus*–penicillin / ampicillin (12 and 10%), and enterococci–ceftiofur / pirlimycin (10% each) combinations, respectively.

Magnitude of the mean bias between Sensititre® automatic and manual MIC readings methods ranged from an underestimation of 15% in *S. aureus*–penicillin combination to an overestimation of 40% in enterococci–pirlimycin combination, respectively (Table 2.6). Limits of agreement varied from an overestimation of 127% to an underestimation of 73% in the former, and from an overestimation of 270% to an underestimation of 46% in the latter.

2.4.3 Agar Disk Diffusion Test Method

The AUC estimates ranged from 0 to 1.00 (Table 2.2). Predictive accuracy of less than 0.5 was evident in 14% of the isolate–antimicrobial combinations. Overall, ADD test method was non–informative, less accurate, moderately accurate, highly accurate and perfect in its predictive accuracy in 3, 7, 36, 20, and 20% of the isolate–antimicrobial combinations, respectively.

Overall categorical agreement was 92%; very major errors, major errors, and discrepancies being 1, 1, and 6% respectively. Very major errors, major errors, and discrepancies ranged from 0 to 20%, 0 to 12%, and 0 to 44% respectively. Enterococci–cephalothin, and *E. coli*–ampicillin combination had the highest percentage of very major errors and discrepancies,

respectively (20 and 44%). Notably, lower categorical agreement values were evident in *E. coli*–cephalothin / ampicillin (56% each), *Strep. dysgalactiae*–tetracycline (55%), and enterococci–cephalothin combinations (47%) (Table 2.5).

2.5 Discussion

The primary objective of this study was to assess diagnostic accuracy of Sensititre® MIC mastitis panel (Sensititre®) and agar disk diffusion method (ADD) of Bauer et al. (1966) with reference to manual broth microdilution test method for antimicrobial resistance profiling of udder pathogens. The study was designed to account for potential variation in susceptibility prevalence due to geographical and epidemiological differences. To the best of knowledge of the authors, Sensititre® and the ADD method have not been compared with a reference manual broth microdilution test method for multiple species of the most common clinical bovine mastitis pathogens.

Quantitative methods of antimicrobial susceptibility testing include agar dilution, broth macrodilution and broth microdilution. Of these, standardized agar dilution test is traditionally considered as the "gold standard" for antimicrobial susceptibility testing (Walker, 2006). However, due to cumbersome nature and lower shelf life of the agar (Schlegelova et al., 2001), and broth macrodilution testing method, broth microdilution is commonly used as a reference method for antimicrobial susceptibility testing. It is an efficient method, and decreased volumes of antimicrobials are used to attain equivalent results with standardized macrodilution method (Gavan and Town, 1970).

In the present study, inter–test off–scale MIC pairs were assumed to be in an essential

agreement. However, the study results would not be valid if the data was not analyzed for finite–scale MICs. Therefore, to avoid bias in the study, data was analyzed for finite–scale, as well for off and on–scale MICs. Further, the data analysis was performed for individual isolate–drug combinations; Hansen and Freedy (1983) made a similar recommendation in their study as well.

Area under the ROC curve was used as a summary estimate of the diagnostic accuracy of Sensititre® and the ADD method in reference to manual broth microdilution test method (Greiner et al., 2000). This estimate indicates the ability of a diagnostic test (Sensititre®/ADD) to discriminate between sensitive and resistant isolates (as determined by reference method) over a range of values of a discriminatory variable (MIC / zone diameter). The AUC estimate can be interpreted as the probability of a higher MIC / lower zone diameter value for a randomly chosen resistant isolate over a sensitive isolate. AUC estimates could not be determined for some isolate–antimicrobial combinations in the present study, as the isolates were either all sensitive or all resistant as determined by the reference method.

Limits of agreement analysis method was employed to quantify precisely, the differences in MICs obtained using Sensititre® automatic and manual readings methods. This method provides a finer approach to compare quantitative agreement between test methods by determining the magnitude of inter–test bias (1 minus mean R value), and thereafter, the limits up to which 95% of the inter–test differences could vary. In certain cases of specific antimicrobial–isolate combinations where the inter–test MIC differences exceeded the acceptable inherent variability range of the MIC dilution systems, the two methods should

not be used interchangeably. This methodology is appropriate to use than the product–moment correlation coefficient (r) method because (r) measures the strength of an association between two methods, and not the agreement (Bland and Altman, 1986). Subsequently, if the LOA values for different isolate–antimicrobial MIC combinations are with in the tolerance range of a single two–fold dilution, Sensititre® automatic and manual reading methods can be used interchangeably.

2.5.1 Sensititre® Automatic MIC Readings Method

Overall, Sensititre® demonstrated a moderate to high predictive accuracy in majority of different isolate–antimicrobial combinations when compared to manual broth microdilution MIC method. There were no ROC AUC estimates for other mastitis pathogen–antimicrobial combinations from previous studies available for comparison.

Sensititre® demonstrated a very high absolute and essential agreement with the reference method for off and on–scale MICs. However, Sensititre® exhibited a profound inclination towards underestimation across different isolate–antimicrobial combinations, for off and on–scale MICs, and finite MICs as well. Even within the tolerance range of MICs, Sensititre® demonstrated increased inclination towards underestimation. Essential agreement between test methods for Gram–positive isolates–beta-lactams combinations were similar to Gavan et al. (1980) (86.7% and 87.8%). However, Sensititre® demonstrated underestimation in 10% of these isolate–antimicrobial combinations in the present study unlike overestimation in the latter study. Lowest essential agreement percentage was evident for *E. coli*–tetracycline combination in the present study (16%); it was far lower

than the lowest essential agreement of 86.7% for staphylococci–penicillin G combination in the study by Jones et al. (1980).

When comparing essential agreement between all Gram–positive and *E. coli* isolates, Sensititre® demonstrated an overall lower essential agreement for the latter. Sensititre® demonstrated consistently lower essential agreement results for *E. coli* isolates as compared to all Gram–positive isolates especially for ampicillin, and cephalothin. Notably, *E. coli*–tetracycline combination had the lowest essential agreement (16%). Furthermore, Sensititre® exhibited higher underestimation proportion in *E. coli*–antimicrobial combinations as compared to all Gram–positive isolates (27% and 9%); on the contrary, no overestimation was evident for the former. Therefore, this bias of Sensititre® with *E. coli* isolates should be kept in mind while performing MIC testing. Even within all Gram–positive isolates category, Sensititre® demonstrated higher essential agreement for *S. aureus* and all streptococcal isolates in comparison to enterococci. Notably, the lowest essential agreement within Gram–positive isolates was evident for enterococci–pirlimycin combination.

Even though the categorical agreement between test methods was very high for most pathogen–antimicrobial combinations, some of the isolate–antimicrobial combinations exhibited a very high percentage of very major errors, notably *E. coli*–cephalothin and *E. coli*–tetracycline (20% and 12%), *Strep. uberis*–pirlimycin (13%), and *Strep. dysgalactiae*–tetracycline and pirlimycin (12% each). Gradus had arbitrated that percentage of very major errors and major errors should be less than 1% and 5%, respectively (1985). Thus susceptibility results from Sensititre® for these pathogen–antimicrobial combinations should be interpreted with caution. Furthermore, Sensititre® exhibited a higher percentage

of categorical agreement results for ampicillin and cephalothin in all Gram–positive isolates than *E. coli,* again demonstrating a bias against *E. coli* microorganisms for such antimicrobial–isolate combinations.

2.5.2 Sensititre® Automatic MIC Readings Test Method in Comparison to Manual MIC Readings Test Method

Except for sulfadimethoxine, overall absolute and essential agreement between test methods for off and on–scale MICs was very high for different isolate–antimicrobial combinations (94% and 97%). Sensititre® manufacturer's instructions for interpretation of sulfadimethoxine MIC states that they must be manually read, thus automatic MIC readings for this antimicrobial are not valid. The lowest absolute agreement was evident in enterococci–ceftiofur and enterococci–pirlimycin combinations. Magnitude of inter-test MIC bias was measured, and the limits of agreement up to which 95% of inter–test MIC differences (or ratio–R, Sensititre® automatic MIC / manual MIC) could vary were determined as well. The acceptable limits of agreement between test methods are single-two fold dilutions (± 1-$\log_2$ dilutions). In the present study, the wider limits of agreement values exceeding the tolerance range of MICs between test methods for *E. coli*-tetracycline, *S. aureus*–ampicillin / penicillin, *Strep. uberis*-pirlimycin / penicillin, *Strep. dysgalactiae*–pirlimycin / erythromycin / tetracycline, and enterococci–ampicillin / oxacillin / ceftiofur / pirlimycin combinations indicate caution while using manual MIC readings. Therefore, manual MIC readings should be avoided, when possible for these isolate–antimicrobial combinations.

2.5.3 Agar Disk Diffusion Method Compared to Manual Broth Microdilution Test Method

Except for few isolate–antimicrobial combinations, the ADD demonstrated a moderate to perfect predictive accuracy across majority of isolate–antimicrobial combinations. However, ADD test method was non–informative for *S. aureus*–oxacillin / ceftiofur, and other streptococci–ampicillin combinations. There were no ROC AUC estimates for other mastitis pathogen–antimicrobial combinations from previous studies available for comparison. Further, the ADD test method demonstrated lower categorical percentage in *E. coli*–ampicillin and *E. coli*–cephalothin combinations in comparison to Gram–positive isolates, a finding similar to Sensititre®, thereby, indicating a biased approach towards these isolate–antimicrobial combinations. Interestingly, very major error percentage for enterococci–various antimicrobials combinations was the highest; slow growth rate of enterococci in MH–agar medium in the ADD test method could be a potential reason for this observation (Hubert et al., 1998). Relatively higher proportions of errors were encountered for various isolate-tetracycline combinations; variation in divalent cations as calcium and magnesium in the MH–agar medium could be a potential reason (CLSI, 2008).

Overall categorical agreement percentage for *S. aureus*–ampicillin / penicillin combination was higher in the present study in comparison to Schlegelova et al. (2001); categorical agreement results for *S. aureus*–cephalothin combination were similar. Relatively higher percentage of agreement for sensitive *S. aureus*–ampicillin / penicillin combination than in for resistant ones was evident in the present study–a finding contrary to Schlegelova et al (2001). Percentage of very major errors in these isolate– antimicrobial combinations was lower in the present study in comparison to the latter.

2.6 Conclusions

Sensititre® demonstrated a range of predictive accuracy between 71 to 99% in 74% of various isolate–antimicrobial combinations; agar disk diffusion method demonstrated a range of predictive accuracy between 71 to 100% in 76% of the isolate–antimicrobial combinations. However, both of these diagnostic tests demonstrated bias against *E. coli* isolates in comparison to the Gram–positive isolates, notably for ampicillin, and cephalothin antimicrobials. Even among Gram-positive isolates, Sensititre® demonstrated higher essential agreement for *S. aureus* and all streptococcal isolates in comparison to enterococci. Caution should therefore be employed while interpreting antimicrobial susceptibility test results in such cases. While similar for most antimicrobial–isolate combinations, Sensititre® automatic readings method is more accurate for most specific isolate–antimicrobial combinations. Overall, both Sensititre® and the agar disk diffusion test method demonstrated higher diagnostic agreement relative to diagnostic accuracy in majority of isolate–antimicrobial combinations.

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Table 2.1: Range of concentrations of antimicrobials used in the manual broth microdilution test method, Sensititre® bovine mastitis panel and commercial agar diffusion disks.

Antimicrobial	Broth microdilution (μg/mL)	Sensititre® (μg/mL)	Agar Diffusion disk (μg/mL)
Ampicillin[1]	0.0075 to 16	0.12 to 8	10
Ceftiofur[2]	0.00375 to 8	0.5 to 4	30
Cephalothin[1]	0.015 to 32	2 to 16	30
Erythromycin[1]	0.00375 to 8	0.25 to 4	15
Oxacillin[1]	0.00375 to 8	2 to 4	1
Penicillin[1]	0.0075 to 16	0.12 to 8	10 IU
Pirlimycin[2]	0.00375 to 8	0.5 to 4	2
Penicillin/Novobiocin[2]	0.00375 to 8	1/2 to 8/16	10 IU/30
Sulfadimethoxine[3]	-	32 to 256	-
Tetracycline[1]	0.0075 to 16	1 to 8	30

[1]Oxoid Canada, Nepean, Ontario.

[2]BD BBL™, Oakville, Ontario.

[3]Rarely used in mastitis treatment and control.

Table 2.2: Diagnostic accuracy estimates of Sensititre® automatic readings (off and on-scale MIC) and agar disk diffusion (ADD) test method with reference to manual broth microdilution test method for clinical bovine mastitis pathogens (n=119).

Isolate (n)[1]	Antimicrobial	Sensititre®	ADD
		AUC[2] (95% CI)[3]	AUC[2] (95% CI)[3]
Escherichia coli (*n* = 25)	Ampicillin	0.71 (0.50 to 0.87)	0.84 (0.63 to 0.95)
	Cephalothin	0.80 (0.59 to 0.93)	0.64 (0.42 to 0.82)
	Tetracycline	0.72 (0.50 to 0.87)	0.92 (0.73 to 0.99)
Staphylococcus aureus (*n* = 24)	Ampicillin	0.87 (0.67 to 0.97)	0.85 (0.62 to 0.95)
	Oxacillin	0.50 (0.29 to 0.70)	0.00 (0.00 to 0.14)[4]
	Penicillin	0.88 (0.67 to 0.97)	0.83 (0.62 to 0.95)
	Ceftiofur	0.27 (0.10 to 0.48)	0.05 (0.00 to 0.21)
Streptococcus uberis (*n* = 20)	Ampicillin	0.95 (0.75 to 0.99)	0.87 (0.62 to 0.96)
	Tetracycline	0.88 (0.68 to 0.98)	0.94 (0.75 to 0.99)
	Pirlimycin	1.00 (0.83 to 1.00)[4]	1.00 (0.83 to 1.00)[4]
Streptococcus dysgalactiae (*n* = 24)	Tetracycline	0.90 (0.73 to 0.98)	0.95 (0.75 to 0.99)
	Pirlimycin	0.94 (0.77 to 0.99)	1.00 (0.85 to 1.00)[4]
Other streptococci (*n* = 11)	Ampicillin	0.44 (0.16 to 0.76)	0.50 (0.23 to 0.83)
	Tetracycline	1.00 (0.71 to 1.00)[4]	1.00 (0.71 to 1.00)[4]
Enterococci (*n* = 15)	Cephalothin	0.98 (0.78 to 1.00)	0.89 (0.59 to 0.98)
	Penicillin	0.93 (0.68 to 0.99)	1.00 (0.78 to 1.00)[4]
	Erythromycin	0.90 (0.68 to 0.99)	0.73 (0.44 to 0.92)

[1]Isolates were either all sensitive or all resistant by manual broth microdilution test method, and hence, no estimates for some antimicrobial-isolate combinations.

[2]AUC: Area under the receiver-operating characteristic (ROC) curve.

[3]CI: 95% binomial exact confidence interval; [4] one-sided, 97.5% confidence interval

Table 2.3: Essential agreement (%) between Sensititre® MIC automatic readings (off and on-scale MIC) and manual broth microdilution MIC test method for clinical bovine mastitis pathogens (n=119).

Isolate (n)	Antimicrobial	R[1]<0.5 (%)	R[1]=0.5 (%)	R[1]=1 (%)	R[1]=2 (%)	R[1]>2 (%)	EA[2] (%)	Errors[3] (%)
Escherichia coli (n=25)	Ampicillin	-	1 (4)	4 (16)	10 (40)	10 (40)	15 (60)	10 (40)
	Oxacillin	-	-	25 (100)	-	-	25 (100)	-
	Cephalothin	-	-	3 (12)	13 (52)	9 (36)	16 (64)	9 (36)
	Penicillin / Novobiocin	-	-	10 (40)	14 (56)	1 (4)	24 (96)	1 (4)
	Erythromycin	-	-	25 (100)	-	-	25 (100)	-
	Tetracycline	-	-	4 (16)	-	21 (84)	4 (16)	21 (84)
	Penicillin	-	-	21 (84)	-	4 (16)	21 (84)	4 (16)
	Ceftiofur	-	-	22 (92)	-	2 (8)	22 (92)	2 (8)
	Pirlimycin	-	-	24 (100)	-	-	24 (100)	-
Staphylococcus aureus (n=24)	Ampicillin	1 (4)	1 (4)	17 (71)	-	5 (21)	18 (75)	6 (25)
	Oxacillin	-	-	23 (96)	-	1 (4)	23 (96)	1 (4)
	Cephalothin	-	-	24 (100)	-	-	24 (100)	-
	Penicillin / Novobiocin	-	-	24 (100)	-	-	24 (100)	-
	Erythromycin	1 (4)	1 (4)	22 (92)	-	-	23 (96)	1 (4)
	Tetracycline	-	-	24 (100)	-	-	24 (100)	-
	Penicillin	2 (8)	-	17 (71)	-	5 (21)	17 (71)	7 (29)
	Ceftiofur	-	5 (22)	15 (61)	3 (13)	1 (4)	23 (96)	1 (4)
	Pirlimycin	-	-	24 (100)	-	-	24 (100)	-
Streptococcus uberis (n=20)	Ampicillin	-	2 (10)	17 (85)	1 (5)	-	20 (100)	-
	Oxacillin	-	-	17 (85)	-	3 (15)	17 (85)	3 (15)
	Cephalothin	1 (5)	-	18 (90)	1 (5)	-	19 (95)	1 (5)
	Penicillin / Novobiocin	-	-	20 (100)	-	-	20 (100)	-
	Erythromycin	-	-	20 (100)	-	-	20 (100)	-
	Tetracycline	1 (5)	-	16 (80)	-	3 (15)	16 (80)	4 (20)
	Penicillin	3 (15)	2 (10)	15 (75)	-	-	17 (85)	3 (15)

	Ceftiofur	-	-	15 (75)	-	5 (25)	15 (75)	5 (25)
	Pirlimycin	-	-	16 (80)	-	4 (20)	16 (81)	4 (20)
Streptococcus dysgalactiae (n=24)	Ampicillin	2 (8)	-	22 (92)	-	-	22 (92)	2 (8)
	Oxacillin	1 (4)	-	23 (96)	-	-	23 (96)	1 (4)
	Cephalothin	3 (12)	-	21 (88)	-	-	21 (88)	3 (12)
	Penicillin / Novobiocin	1 (4)	-	23 (96)	-	-	23 (96)	1 (4)
	Erythromycin	3 (12)	-	21 (88)	-	-	21 (88)	3 (12)
	Tetracycline	1 (4)	-	8 (33)	6 (25)	9 (38)	14 (58)	10 (42)
	Penicillin	2 (8)	-	22 (92)	-	-	22 (92)	2 (8)
	Ceftiofur	-	-	24 (100)	-	-	24 (100)	-
	Pirlimycin	1 (4)	-	20 (84)	-	3 (12)	20 (84)	4 (16)
Other streptococci (n=11)	Ampicillin	1 (9)	-	8 (73)	-	2 (18)	8 (73)	3 (27)
	Oxacillin	-	-	11 (100)	-	-	11 (100)	-
	Cephalothin	-	1 (9)	9 (82)	-	1 (9)	10 (91)	1 (9)
	Penicillin / Novobiocin	-	-	11 (100)	-	-	11 (100)	-
	Erythromycin	-	-	11 (100)	-	-	11 (100)	-
	Tetracycline	-	-	10 (91)	1 (9)	-	11 (100)	-
	Penicillin	-	1 (9)	10 (91)	-	-	11 (100)	-
	Ceftiofur	-	-	10 (91)	-	1 (9)	10 (91)	1 (9)
	Pirlimycin	-	-	11 (100)	-	-	11 (100)	-
Enterococci (n=15)	Ampicillin	-	1 (7)	7 (46)	3 (20)	4 (27)	11 (73)	4 (27)
	Oxacillin	-	-	2 (13)	8 (54)	5 (33)	10 (67)	5 (33)
	Cephalothin	-	1 (7)	6 (40)	7 (46)	1 (7)	14 (93)	1 (7)
	Penicillin / Novobiocin	-	6 (40)	3 (20)	5 (33)	1 (7)	14 (93)	1 (7)
	Erythromycin	-	-	6 (40)	7 (46)	2 (13)	13 (87)	2 (13)
	Tetracycline	-	1 (7)	2 (13)	11 (73)	1 (7)	14 (93)	1 (7)
	Penicillin	-	4 (27)	6 (40)	2 (13)	3 (20)	12 (80)	3 (20)
	Ceftiofur	-	-	10 (66)	1 (7)	4 (27)	11 (73)	4 (27)
	Pirlimycin	1 (7)	-	7 (46)	1 (7)	6 (40)	8 (53)	7 (47)
Overall		25 (2)	27 (3)	806 (75)	94 (9)	117 (11)	927 (87)	142 (13)

[1]R: Manual broth microdilution MIC/ Sensititre® automatic MIC ratio; R=1 indicates absolute inter-test MIC agreement (no difference between Manual broth microdilution MIC and Sensititre® automatic MIC value); R=0.5 to 2 indicates acceptable inherent variability (tolerance) range in MIC dilution systems; R<0.5 & R>2 indicates respective over and underestimation by Sensititre® in reference to manual broth microdilution test method.

[2]EA: Essential agreement (Percentage of manual broth microdilution MIC and Sensititre® automatic MIC pairs within tolerance range).

[3]Errors: Percentage of manual broth microdilution MIC and Sensititre® automatic MIC pairs out of tolerance range.

Table 2.4: Lower essential agreement (%) between Sensititre® MIC automatic readings (off and on-scale MIC) and manual broth microdilution MIC test method for *Escherichia coli* isolates in comparison to Gram-positive bovine clinical mastitis isolates (n=119).

Antimicrobial	*Staphylococcus aureus*	*Streptococcus uberis*	*Streptococcus dysgalactiae*	Other Streptococci	*Enterococci*	All Streptococci[1]	Gram + Isolates[2]	*Escherichia coli*
Ampicillin	75	100	92	73	73	88	83	60
Oxacillin	96	85	96	100	67	94	89	100
Cephalothin	100	95	88	91	93	91	93	64
Penicillin / Novobiocin	100	100	96	100	93	99	98	96
Erythromycin	96	100	88	100	87	96	94	100
Tetracycline	100	80	58	100	93	79	86	16
Penicillin	71	85	92	100	80	92	86	84
Ceftiofur	96	75	100	91	73	89	87	92
Pirlimycin	100	81	84	100	53	88	84	100
Overall	93	89	88	95	79	91	89	79

[1]All Streptococcal isolates include *Strep. uberis*, *Strep. dysgalactiae*, and other Streptococci.

[2]All Gram-positive isolates include *S. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, other Streptococci, and *enterococci*.

Table 2.5: Categorical agreement (%) between Sensititre® automatic readings (off and on-scale MIC) and agar disk diffusion (ADD) test method with reference to manual broth microdilution test method for clinical bovine mastitis pathogens (n=119).

Isolates (n)	Sensititre®				Agar Disk Diffusion			
	CA[1]	VME[2]	ME[3]	D[4]	CA[1]	VME[2]	ME[3]	D[4]
Escherichia coli (25)								
Ampicillin	18 (72)	2 (8)	-	5 (20)	14 (56)	-	-	11 (44)
Cephalothin	8 (32)	5 (20)	-	12 (48)	14 (56)	2 (8)	1 (4)	8 (32)
Tetracycline	20 (80)	3 (12)	-	2 (8)	22 (88)	1 (4)	-	2 (8)
Ceftiofur	25 (100)	-	-	-	25 (100)	-	-	-
Staphylococcus aureus (24)								
Ampicillin	22 (92)	2 (8)	-	-	22 (92)	1 (4)	1 (4)	-
Oxacillin	23 (96)	1 (4)	-	-	23 (96)	1 (4)	-	-
Cephalothin	24 (100)	-	-	-	24 (100)	-	-	-
Penicillin/Novobiocin	24 (100)	-	-	-	24 (100)	-	-	-
Erythromycin	24 (100)	-	-	-	20 (83)	-	-	4 (17)
Tetracycline	24 (100)	-	-	-	24 (100)	-	-	-
Penicillin	21 (88)	1 (4)	2 (8)	1 (4)	23 (96)	-	-	1 (4)
Ceftiofur	23 (96)	-	-	1 (4)	23 (96)	-	-	1 (4)
Pirlimycin	24 (100)	-	-	-	24 (100)	-	-	-
Streptococcus uberis (20)								
Ampicillin	19 (95)	-	-	1 (5)	18 (90)	-	-	2 (10)
Cephalothin	20 (100)	-	-	-	20 (100)	-	-	-
Penicillin/Novobiocin	20 (100)	-	-	-	20 (100)	-	-	-
Erythromycin	20 (100)	-	-	-	20 (100)	-	-	-
Tetracycline	16 (80)	2 (10)	1 (5)	1 (5)	16 (80)	-	1 (5)	3 (15)
Penicillin	20 (100)	-	-	-	20 (100)	-	-	-
Ceftiofur	20 (100)	-	-	-	20 (100)	-	-	-
Pirlimycin	17 (87)	3 (13)	-	-	20 (100)	-	-	-
Streptococcus dysgalactiae (24)								
Ampicillin	22 (92)	-	1 (4)	1 (4)	24 (100)	-	-	-
Cephalothin	23 (96)	-	1 (4)	-	24 (100)	-	-	-
Penicillin/Novobiocin	23 (96)	-	1 (4)	-	24 (100)	-	-	-
Erythromycin	21 (88)	-	2 (8)	1 (4)	24 (100)	-	-	-
Tetracycline	12 (50)	3 (12)	-	9 (37)	13 (55)	-	3 (12)	8 (33)
Penicillin	24 (100)	-	-	-	24 (100)	-	-	-
Ceftiofur	24 (100)	-	-	-	24 (100)	-	-	-
Pirlimycin	20 (84)	3 (12)	1 (4)	-	24 (100)	-	-	-

Isolates (n)	Sensititre®				Agar Disk Diffusion			
	CA[1]	VME[2]	ME[3]	D[4]	CA[1]	VME[2]	ME[3]	D[4]
Other streptococci (11)								
Ampicillin	9 (82)	-	-	2 (18)	9 (82)	-	-	2 (18)
Cephalothin	11 (100)	-	-	-	11 (100)	-	-	-
Penicillin/Novobiocin	11 (100)	-	-	-	11 (100)	-	-	-
Erythromycin	11 (100)	-	-	-	11 (100)	-	-	-
Tetracycline	10 (91)	-	-	1 (9)	10 (91)	-	-	1 (9)
Penicillin	11 (100)	-	-	-	11 (100)	-	-	-
Ceftiofur	11 (100)	-	-	-	11 (100)	-	-	-
Pirlimycin	11 (100)	-	-	-	11 (100)	-	-	-
Enterococci (15)								
Ampicillin	15 (100)	-	-	-	14 (93)	-	-	1 (7)
Cephalothin	13 (86)	1 (7)	-	1 (7)	7 (47)	3 (20)	-	5 (33)
Erythromycin	13 (86)	-	-	2 (14)	11 (73)	-	-	4 (27)
Penicillin	14 (93)	1 (7)	-	-	13 (86)	2 (14)	-	-
Overall	740 (91)	27 (3)	9 (1)	40 (5)	747 (92)	11 (1)	6 (1)	52 (6)

[1]CA: Categorical agreement (Accordance for sensitive and resistant isolates between Sensititre® automatic readings, agar disk diffusion test method with reference to manual broth microdilution test method).

[2]VME: Very major error (An isolate categorized as resistant by reference method, but classified as sensitive by Sensititre® automatic readings / agar disk diffusion test method (false-sensitive)).

[3]ME: Major error (An isolate categorized as sensitive by reference method, but classified as resistant by Sensititre® automatic readings / agar disk diffusion test method (false-resistant)).

[4]D: Discrepancy (An intermediate isolate classified as sensitive or resistant and vice-verse).

Table 2.6: Differences between Sensititre® automatic MIC readings (off and on-scale MIC) and Sensititre® manual MIC readings test method for clinical bovine mastitis pathogens (n=457).

Isolate (n)	Antimicrobial	Mean R[1] (95% CI)	Bias (%)[2]	Limits of R[3]	Limits of Agreement[4]
Escherichia coli (153)	Ampicillin	1 (0.98 to 1.02)	-	0.76 to 1.30	31 (+) to 24 (-)
	Oxacillin	1 (1 to 1)	-	1 to 1	-
	Cephalothin	0.96 (0.93 to 0.99)	4 (+)	0.66 to 1.40	51 (+) to 28 (-)
	Penicillin/Novobiocin	0.94 (0.92 to 0.97)	6 (+)	0.65 to 1.37	53 (+) to 27 (-)
	Ceftiofur	0.99 (0.99 to 1.00)	1 (+)	0.89 to 1.10	12 (+) to 10 (-)
	Pirlimycin	1 (1 to 1)	-	1 to 1	-
	Erythromycin	1 (1 to 1)	-	1 to 1	-
	Tetracycline	0.91 (0.86 to 0.96)	9 (+)	0.45 to 1.80	122 (+) to 44 (-)
	Penicillin	0.98 (0.97 to 1.00)	2 (+)	0.81 to 1.19	23 (+) to 16 (-)
Staphylococcus aureus (151)	Ampicillin	1.13 (1.06 to 1.22)	11 (-)	0.47 to 2.74	112 (+) to 63 (-)
	Oxacillin	1 (1 to 1)	-	1 to 1	-
	Cephalothin	1 (1 to 1)	-	1 to 1	-
	Penicillin/Novobiocin	1 (1 to 1)	-	1 to 1	-
	Ceftiofur	0.98 (0.96 to 1.00)	2 (+)	0.71 to 1.34	40 (+) to 26 (-)
	Pirlimycin	0.99 (0.98 to 1.00)	1 (+)	0.85 to 1.16	17 (+) to 14 (-)
	Erythromycin	1.04 (1.00 to 1.08)	3 (-)	0.63 to 1.71	58 (+) to 41 (-)
	Tetracycline	1.04 (1.00 to 1.08)	4 (-)	0.63 to 1.71	58 (+) to 41 (-)
	Penicillin	1.17 (1.08 to 1.27)	15 (-)	0.44 to 3.70	127 (+) to 73 (-)
Streptococcus uberis (47)	Ampicillin	1.04 (0.98 to 1.11)	4 (-)	0.66 to 1.64	51 (+) to 39 (-)
	Oxacillin	0.94 (0.86 to 1.02)	6 (+)	0.53 to 1.66	88 (+) to 39 (-)
	Cephalothin	1.10 (0.99 to 1.22)	10 (-)	0.54 to 2.24	85 (+) to 55 (-)
	Penicillin/Novobiocin	1 (1 to 1)	-	1 to 1	-
	Ceftiofur	1 (1 to 1)	-	1 to 1	-
	Pirlimycin	0.74 (0.63 to 0.88)	35 (+)	0.23 to 2.33	334 (+) to 58 (-)

	Erythromycin	1.05 (0.98 to 1.15)	4 (-)	0.60 to 1.85	66 (+) to 45 (-)
	Tetracycline	0.98 (0.96 to 1.01)	2 (+)	0.81 to 1.19	23 (+) to 16 (-)
	Penicillin	1.16 (1.02 to 1.31)	14 (-)	0.50 to 2.66	100 (+) to 63 (-)
Streptococcus dysgalactiae (49)	Ampicillin	1.05 (0.98 to 1.15)	5 (-)	0.60 to 1.85	66 (+) to 45 (-)
	Oxacillin	1 (1 to 1)	-	1 to 1	-
	Cephalothin	1.11 (1 to 1.24)	9 (-)	0.52 to 2.41	92 (+) to 58 (-)
	Penicillin/Novobiocin	1 (1 to 1)	-	1 to 1	-
	Ceftiofur	0.98 (0.92 to 1.02)	2 (+)	0.65 to 1.43	53 (+) to 30 (-)
	Pirlimycin	0.89 (0.81 to 0.99)	12 (+)	0.44 to 1.82	127 (+) to 46 (-)
	Erythromycin	1.05 (0.88 to 1.25)	4 (-)	0.30 to 3.70	233 (+) to 72 (-)
	Tetracycline	0.98 (0.88 to 1.09)	2 (+)	0.45 to 2.13	122 (+) to 54 (-)
	Penicillin	1.10 (0.98 to 1.24)	10 (-)	0.48 to 2.50	108 (+) to 60 (-)
Other streptococci (18)	Ampicillin	1 (1 to 1)	-	1 to 1	-
	Oxacillin	1 (1 to 1)	-	1 to 1	-
	Cephalothin	1 (1 to 1)	-	1 to 1	-
	Penicillin/Novobiocin	1 (1 to 1)	-	1 to 1	-
	Ceftiofur	0.96 (0.88 to 1.04)	4 (+)	0.69 to 1.33	44 (+) to 25 (-)
	Pirlimycin	0.93 (0.78 to 1.08)	7 (+)	0.48 to 1.76	108 (+) to 44 (-)
	Erythromycin	1 (1 to 1)	-	1 to 1	-
	Tetracycline	1 (1 to 1)	-	1 to 1	-
	Penicillin	1 (1 to 1)	-	1 to 1	-
Enterococci (29)	Ampicillin	0.86 (0.77 to 0.97)	16 (+)	0.49 to 1.52	104 (+) to 34 (-)
	Oxacillin	0.76 (0.61 to 0.95)	31 (+)	0.24 to 2.39	316 (+) to 58 (-)
	Cephalothin	0.93 (0.86 to 1.01)	7 (+)	0.60 to 1.41	66 (+) to 29 (-)
	Penicillin/Novobiocin	0.97 (0.92 to 1.02)	3 (+)	0.75 to 1.26	33 (+) to 20 (-)
	Ceftiofur	0.86 (0.72 to 1.03)	16 (+)	0.33 to 2.20	203 (+) to 55 (-)
	Pirlimycin	0.71 (0.60 to 0.86)	40 (+)	0.27 to 1.84	270 (+) to 46 (-)
	Erythromycin	0.93 (0.83 to 1.03)	7 (+)	0.53 to 1.63	88 (+) to 38 (-)
	Tetracycline	0.97 (0.92 to 1.02)	3 (+)	0.75 to 1.26	33 (+) to 20 (-)
	Penicillin	0.91 (0.83 to 0.99)	9 (+)	0.55 to 1.47	81 (+) to 32 (-)

[1]Mean R: Mean of Sensititre® automatic MIC / manual MIC ratios.

[2]Absolute Bias: Average over-estimation (+) or under-estimation (-) percentage by Sensititre® manual MIC readings method when compared to Sensititre® automatic MIC readings method.

[3]Limits of R: Range of Sensititre® automatic MIC / manual MIC ratios.

[4]LOA: Limits of over-estimation (+) and / or under-estimation (-) percentage by Sensititre® manual MIC test method when compared to Sensititre® automatic MIC readings method

Chapter Three: Antimicrobial use on Canadian dairy farms

3.1 Abstract

Antimicrobial use (AMU) data are critical for formulating policies for containing antimicrobial resistance. The present study determined AMU on Canadian dairy farms and characterized variation in AMU based on herd-level factors such as milk production, somatic cell count, herd size, geographic region and housing type. Drug use data were collected on 89 dairy herds in 4 regions of Canada (Alberta, Ontario, Québec and the Maritime provinces [Prince Edward Island, New Brunswick, Nova Scotia]) for an average of 540 days per herd. Dairy producers and farm personnel were asked to deposit empty drug containers into specially provided receptacles. Antimicrobial use was measured as antimicrobial drug use rate (ADUR) with the unit being number of animal defined-daily doses (ADD)/1000 cow-days. Antimicrobial drug use rates were determined at farm, region and national level.

Combined ADUR of all antimicrobial classes was 14.35 ADD/1000 cow-days nationally. National level ADUR of the 6 most commonly used antimicrobial drug classes, cephalosporins, penicillins, penicillin combinations, tetracyclines, trimethoprim-sulfonamide combinations and lincosamides were 3.05, 2.56, 2.20, 1.83, 0.87 and 0.84 ADD/1000 cow-days, respectively. Dairy herds in Ontario were higher users of third-generation cephalosporins (ceftiofur) than in Québec. Alberta dairy herds were higher users of tetracyclines in comparison to Maritimes. ADUR was higher via systemic route as compared to intramammary and other routes of administration (topical, oral, and intrauterine). ADUR of antimicrobials used intramammarily was higher for clinical mastitis

treatment than dry cow therapy. For dry cow therapy, penicillin ADUR was greater than ADUR of first-generation cephalosporins. For clinical mastitis treatment, ADUR of intramammary penicillin combinations was greater than ADUR of cephapirin. Herd-level milk production was positively associated with overall ADUR, ADUR of systemically administered ceftiofur, cephapirin administered for dry cow therapy, and pirlimycin administered for clinical mastitis treatment. Herd size and ADUR of systemically administered ceftiofur were also positively associated.

In conclusion, ß-lactams were most commonly used on Canadian dairy farms. Among antimicrobials of very high importance in human medicine, the use of fluoroquinolones was rare, whereas third-generation cephalosporins and penicillin combinations containing colistin were used very frequently.

3.2 Introduction

The importance of antimicrobials in human and veterinary medicine for disease prevention and control cannot be understated (Morley et al., 2005). Not only are antimicrobials indispensible tools for decreasing morbidity and mortality due to infectious diseases, their use in veterinary medicine had a profound impact on animal health and productivity (Johnston, 1998). The commercial production and use of antimicrobials after the late 1940s in animal agriculture resulted in the effective treatment of infections, previously considered being untreatable (Prescott, 2006). Unfortunately, emergence and dissemination of antimicrobial resistance (AMR) has followed the introduction of antimicrobials.

Various national and international bodies are intensely scrutinizing the factors promoting the emergence and dissemination of resistance among microbial pathogens in humans and animals alike (CODEX, 2005; WHO, 2000). Decades of use and misuse of antimicrobials in human and veterinary medicine is considered to be the primary factor responsible for emergence of resistance in bacteria (Levy and Marshall, 2004). Although antimicrobial use (AMU) and overuse is common in human medicine (Paskovaty et al., 2005), there is an increased focus on veterinary AMU especially in food animals as a potential source and disseminator of AMR in bacteria infecting humans (White and McDermott, 2001); the concern being the use of relatively larger quantities, and the similarity to antimicrobial drug classes used in human medicine (Silbergeld et al., 2008; Veterinary Drug Directorate, 2002). Despite the lack of scientifically sound information about adverse health aspects due to AMU in food animals (Phillips et al., 2004), global pressure to protect the efficacy of

existing and new antimicrobials by reducing the selection pressure on bacterial populations for emergence of resistant strains due to AMU is mounting. In order to understand the public health risks associated with the use of antimicrobials in food animals, quantitative assessment of AMU in food animals is imperative for determining AMR epidemiology. The availability of AMU data can aid in interpreting patterns and trends of AMR, serve as a basis of risk assessment of AMR, as a basis of decision-making for control measures, and to evaluate the impact of interventions for controlling AMR (WHO, 2003). Data on AMU in food animals are therefore becoming increasingly important for developing national and international policies to contain AMR.

International bodies such as the Food and Agriculture Organization, the Office International des Épizooties and the World Health Organization have recommended implementing on-going and coordinated national surveillance programs for assessing AMU in food animals (Nicholls et al., 2001; WHO, 2003). Various countries including Denmark, The Netherlands, Norway, Sweden, and UK have therefore implemented AMU and AMR monitoring programs. In North America, the United States has started collecting data on AMU in dairy cattle through various surveys (Pol and Ruegg, 2007; Sawant et al., 2005; Zwald et al., 2004). Data on AMU in food animals in Canada are limited mainly to swine and beef herds (Carson et al., 2008; Dunlop et al., 1998; Gow and Waldner, 2009). Except a study conducted by Meek et al. (1986) to assess AMU on dairy farms in Ontario, there is a lack of information on AMU on Canadian dairy farms. Recently, the Canadian Integrated Program for Antimicrobial Resistance Surveillance has started collaborating with various universities to assess AMU and resistance in dairy cattle in Canada (CIPARS, 2007).

The objectives of the present study were to describe the qualitative and quantitative aspects of AMU on Canadian dairy farms and to assess the association between AMU and herd-level factors such as milk production, somatic cell count, herd size, geographic region and housing type.

3.3 Materials and Methods

3.3.1 Dairy Cattle Herd Selection

Data for this study originated from the National Cohort of Dairy Farms of the Canadian Bovine Mastitis Research Network, which consisted of 91 commercial dairy farms located in 4 regions across Canada (Alberta, Québec, Ontario and the Maritime Provinces [Prince Edward Island, Nova Scotia, and New Brunswick]). Herd selection criteria for the present study have been described by Reyher et al. (2011). In short, dairy herds were selected to replicate the provincial proportion of free-stall systems to within 15 percentage points and to be uniformly distributed among three strata of the most recent 12-month bulk tank SCC average (≤ 150,000 cells/mL, > 150,000 and ≤ 300,000 cells/mL, > 300,000 cells/mL); herds with a 3X milking schedule and herds with less than 80% (or less than 15) Holstein lactating and dry cows at the time of enrolment were excluded. Further, eligible dairy herds must have been participating in a DHI data collection program. Eligible dairy farms were identified and contacted by the regional center coordinators. Written consent to participate in the research cohort was obtained. Two dairy herds dropped out of the study at the very beginning. Average herd size of the 89 participating dairy herds was 84 cows (median: 66, range: 33 – 297). Herd average daily milk production per cow ranged from 25 to 39kg (mean and median: 32kg). Average herd SCC ranged from 91,000 – 500,000 cells/mL (arithmetic mean: 230,000 cells/mL; median: 220,000 cells/mL). Sixty-one, 33, 5 and 1%

of these dairy herds housed lactating cows in tie-stalls, free-stalls, in a bedding packed barn, and mixed barn type, respectively. On the regional level, dairy herds in Alberta had the largest average herd size (mean: 110, median: 99, range: 42 – 297) followed by Maritimes (mean: 87, median: 66, range: 52 – 234), Ontario (mean: 80, median: 67, range: 33 – 182), and Québec (mean: 68, median: 60, range: 33 – 184). Herd average daily milk production per cow (kg/cow) was similar in dairy herds in Alberta (mean: 33, median: 33, range: 27 – 38) and Ontario (mean: 33, median: 33, range: 26 – 38), followed by Maritimes (mean: 32, median: 31, range: 28 – 39), and Québec (mean: 30, median: 31, range: 25 – 34). Average herd SCC was the highest in dairy herds in Ontario (arithmetic mean: 249,000 cells/mL, median: 244,000 cells/mL, range: 93,000 – 500,000 cells/mL), followed by Québec (arithmetic mean: 245,000 cells/mL, median: 229,000 cells/mL, range: 91,000 – 467,000 cells/mL), Alberta (arithmetic mean: 218,000 cells/mL, median: 179,000 cells/mL, range: 116,000 – 355,000 cells/mL), and Maritimes (arithmetic mean: 189,000 cells/mL, median: 162,000 cells/mL, range: 95,000 – 416,000 cells/mL). An investigator and technicians in each coordinating center were responsible for the data collection activities related to farms located in that center's area.

3.3.2 Antimicrobial Use Data Collection Methodology

Antimicrobial use data were collected from February 2007 until December 2008. Forty-liter receptacles with round swing tops (Sterlite) were placed on participating farms for collecting data for AMU. These receptacles were labeled as " Drug containers only" and were placed near the drug storage area, in the milking parlour or any place near where the treatments were normally given (near the chute, for example). Producers, farm workers and other farm personnel were instructed to deposit the empty containers of all drugs used by

him/her or the veterinarian for treatment in calves, heifers and adult cows (dry cows and lactating cows) into these receptacles. These containers included all empty drug bottles, drug containers, lactating cow or dry cow intramammary tubes, tablet and powder containers, medicated milk replacer and feed tags and any other drug containers used on the farms. Any remaining drug products were also placed in the receptacles. In case a drug bottle was broken, producers were instructed to put the label into the receptacle instead. Farms were visited at least once per month. The technician and students would empty the receptacle, count the empty drug bottles and record the inventory in the drug tally sheets at the dairy farm. The drug tally sheets contained information on the following: herd identification number, start and end date of the current data collection period, date of tallying drugs, product name, volume or weight of the product, and number of containers deposited in the receptacle during current data collection period.

3.3.3 Statistical Analyses

Drug use data were entered into a customized database (Microsoft Office Access 2006, Microsoft Corporation, Redmond, Washington, USA). A random sample of the drug tally sheets (25%) was checked manually to detect errors in data entry. Data analyses were performed using Intercooled Stata®11.1 (Intercooled Stata for Macintosh, version 11.1, Stata Corporation, College Station, TX).

Antimicrobial use data were quantified in units of animal defined-daily doses (ADD). The ADD (g/day) was defined as the average daily on-label dosage multiplied by the approximate weight of an adult dairy cow (BW=600 kg) (Jensen et al., 2004) and was based on the Canadian compendium of veterinary products (CVP). Animal defined-daily

doses for antimicrobials indicated for heifers and calves were calculated for body weight of 200 and 50 kg, respectively. In case of trimethoprim (TMP)-sulfonamide combinations, the ADD was calculated on the basis of TMP (the constituent drug of interest) as per Grave et al. (1999). For the remaining combination compounds, e.g. penicillin combinations (intramammary preparations containing penicillin G procaine – dihydrostreptomycin sulfate – novobiocin sodium – polymyxin B sulfate), the weights of active ingredients of the constituents were summarized to determine total weight of active ingredients in the combination compound. Amount of active ingredients given in IU were converted into mg as follows: 1000 IU of penicillin G procaine = 0.6 mg, 1000 IU of polymyxin B = 0.1 mg (Prescott and Dowling, 2006). Further, antimicrobial drug use rate (ADUR) – a herd-level and time-sensitive parameter of AMU – was defined as number of ADD used on a farm per 1,000 cows per day:

$$\text{ADUR} = \frac{\text{Active ingredient used in the study period (g)} \times 1000}{\text{ADD} \times \text{Number of adult cows} \times \text{Number of days in the study period}}$$

Numbers of days of drug use on a dairy farm in a data collection period were calculated from the starting and end date on a drug tally sheet. Total number of adult cattle (dry and lactating) at the dairy farm in the study period was also determined concurrently. Because total number of adult cattle varied very little from month to month (as determined by DHI test day data at each farm), an average of total number of adult cattle per farm was calculated and multiplied with total number of days of drug use at that farm to determine farm level cow-days. Within a region, farm level cow-days of various farms were

aggregated to obtain region level cow-days, and later region level cow-days were aggregated to obtain national level cow-days.

ADUR of various antimicrobial drug classes were determined at farm, region and national level. Overall ADUR – the combined ADUR of all antimicrobial classes – was also determined at these 3 levels. Overall ADUR at farm, region and national level were estimated by dividing combined ADD of all antimicrobial drug classes used at a farm, in a region and at national level by farm, region and national level cow-days, respectively. Region level overall ADUR was the regional estimate of AMU, whereas national level overall ADUR was the national estimate of AMU. Data from Maritime Provinces (Prince Edward Island, Nova Scotia, and New Brunswick) were combined due to regional homogeneity so as to facilitate statistical analysis.

At the national level, statistical significance of differences in the number of ADD among various antimicrobial classes was evaluated by chi-square test. Thereafter, 95% confidence intervals around ADD were calculated. Non-overlapping 95% confidence intervals indicated statistically significant differences in ADUR among various antimicrobial classes at the national level.

Statistical significance of variation in ADUR of various antimicrobial classes was determined across 4 regions using Kruskal Wallis test (Null hypothesis: no differences in ADUR across 4 regions; Alternative hypothesis: at least one of the regions is different). In case of statistically significant result, pair-wise comparisons between regions were done using Wilcoxon rank-sum test to determine pairs of regions that were statistically different.

Superscripts were placed on the herds within regions in the tables; pairs of regions with common superscripts differed significantly in the distributions of ADUR from each other. Lastly, variation in ADUR within 4 regions was determined by evaluating differences in the number of ADD of various antimicrobial classes using chi-square test and 95% confidence intervals. Again, non-overlapping 95% confidence intervals indicated statistically significant differences in ADUR of various antimicrobial classes within 4 regions.

The differences between ADUR of antimicrobial drug classes administered by the intramammary route for dry cow therapy and clinical mastitis treatment were determined using the Wilcoxon matched-pairs signed-ranks test. Pair-wise comparisons of ADUR between barn types (tie-stalls and free-stalls) were done using the Wilcoxon rank-sum test. Correlation between herd-level ADUR and average herd-level milk production, average herd SCC and herd size was estimated using Spearman's rank correlation coefficient. Bonferroni adjustments were done whenever multiple comparisons were made (Abdi, 2007). In all other instances, a P-value < 0.05 was considered statistically significant.

3.4 Results

3.4.1 Antimicrobial Drug Use Rates

Among the antimicrobial drug classes used, cephalosporins (cephapirin, ceftiofur), penicillins (ampicillin, amoxicillin, penicillin G procaine, penicillin G procaine – penicillin G benzathine combination, penicillin G procaine – novobiocin combination), penicillin combinations (intramammary preparation containing penicillin G procaine –

dihydrostreptomycin sulfate – novobiocin sodium – polymyxin B sulfate combination), TMP-sulfonamide combinations (TMP-sulfadoxine), tetracyclines (oxytetracycline and tetracycline hydrochloride) and lincosamides (pirlimycin) were used on the majority of the participating dairy farms (Table 3.1). At national level, cephalosporins were the antimicrobials with the highest ADUR, followed by penicillins, penicillin combinations, tetracyclines, TMP-sulfonamide combinations and lincosamides in declining order.

3.4.2 Region

ADUR of tetracyclines, third-generation cephalosporins (ceftiofur) and penicillins was the highest among drug classes used in Alberta, Ontario, and Québec and Maritimes, respectively (Table 3.2). Differences in the distribution of ADUR of third-generation cephalosporins (ceftiofur) and tetracyclines between regions were observed. Dairy herds in Ontario were significantly higher users of third-generation cephalosporins (ceftiofur) than in Québec. Alberta dairy herds were significantly higher users of tetracyclines in comparison to Maritimes.

3.4.3 Route of Administration

At the national level, systemically administered antimicrobials had the highest ADUR followed by ADUR of antimicrobials administered via intramammary and, finally, antimicrobials administered by other routes (topical, oral and intrauterine) (5.46, 5.09 and 3.79, respectively). Similar differences were evident in Alberta and Ontario as well, with the exception of Québec where ADUR of intramammary antimicrobials was greater than ADUR of systemically administered antimicrobials and antimicrobials administered by other routes (Table 3.3). Further, differences in ADUR between systemic and

intramammary routes were not observed for Maritimes dairy herds.

3.4.4 Intramammary

At the national level, penicillin combinations had the highest intramammary ADUR followed by penicillins, first-generation cephalosporins (cephapirin) and lincosamides (2.20, 1.28, 0.83 and 0.66, respectively) (Table 3.4). Intramammary ADUR of penicillins used for dry cow therapy was significantly higher in Québec than in Ontario (Table 3.5). Maritimes dairy herds were significantly higher users of intramammary first-generation cephalosporins (cephapirin) than Alberta.

3.4.5 Systemic

At national level, ß-lactams were the most common and fluoroquinolones were the least commonly used systemic antimicrobial drug classes, being used on 88/89 and 4/89 dairy farms, respectively. Third-generation cephalosporins (ceftiofur) had the highest ADUR followed by penicillins, TMP-sulfonamide combinations and tetracyclines. Regional differences for systemic antimicrobial drugs are presented in Table 3.6. Dairy herds in Québec were significantly lower users of systemic ceftiofur than Ontario and Maritimes. Further, dairy herds in Maritimes were significantly lower users of systemic tetracyclines than in Alberta.

3.4.6 Herd-level Factors

Average herd milk production and herd-level overall ADUR were positively correlated ($P < 0.05$, Table 3.7, Fig. 3.1). But, except for ADUR of systemic third-generation cephalosporins (ceftiofur), intramammary first-generation cephalosporins (cephapirin)

administered for dry cow therapy and lincosamides administered for clinical mastitis treatment, correlations between average herd milk production and herd-level ADUR of specific antimicrobial drug classes were not significant and ranged from – 0.16 to 0.16 (P > 0.10 in all instances). Average herd SCC and herd-level overall ADUR were also not significantly correlated. Average herd size and use of systemic third-generation cephalosporins (ceftiofur) was positively correlated. Otherwise, average herd size and herd-level overall ADUR were not significantly correlated.

Herd-level overall ADUR was not significantly different between farms using tie-stalls and farms using free-stalls (11.03 and 16.66, respectively, P = 0.45). ADUR of systemically administered penicillins was greater in tie-stalls than in free-stalls; however, the differences were not significant (median: 1.34 and 0.61, respectively; P = 0.06). Similarly, ADUR of intramammary penicillin combinations used for clinical mastitis treatment was also greater in tie-stalls than in free-stalls; however differences were also not significant (median: 1.85 and 1.43, respectively, P = 0.06).

3.5 Discussion

The present study was conducted to determine AMU on Canadian dairy farms and to identify herd-level factors that characterize variation in AMU. This was the first time that such a Canada-wide prospective study was undertaken to quantify use of antimicrobials on dairy farms. Antimicrobial drug use rate was an estimate of AMU. In order to compare ADUR between different farms, it was assumed that antimicrobials were used as per directions on the label, all replacement animals were born and raised on the farm (Pol and Ruegg, 2007), and that herd size remained constant over the study period.

ADUR data were highly skewed, and therefore, non-parametric tests were used to determine statistical significance of differences in the distribution of ADUR of various antimicrobial classes. Kruskal Wallis and Wilcoxon rank-sum tests compared entire distributions of ADUR across 4 regions and between pairs of regions, respectively, rather than a particular test statistic as mean or median. Medians were reported to indicate the direction of differences in the skewed distributions. Spearman's rank correlation coefficient, a non-parametric measure of statistical dependence between the ranked variables, was estimated to determine correlations between herd-level ADUR and average herd-level milk production, average herd SCC and herd size.

Various sources of determining information on AMU in food animal production systems include wholesalers, pharmacists, veterinarians, feed companies and animal producers; accessibility and accuracy of the information collected as such depends upon the objectives of the study and data available (Chauvin et al., 2001). Methods used commonly for measuring on-farm AMU include mailing out cross-sectional surveys to producers (Sawant et al., 2005; Spicer et al., 1994), filling in of treatment diaries by dairy producers (Meek et al., 1986), and inventory of empty antimicrobial containers (Carson et al., 2008). However, inadequate response to the self-reported surveys, recall bias, producer non-compliance, under recording/incomplete/inaccurate/unverifiable recording in the treatment diaries by producers are some of the issues associated with methods of AMU measurement (Raymond et al., 2006; Zwald et al., 2004; Sawant et al., 2005; Pol and Ruegg, 2007; Kaneene and Ahl, 1987). Due to difficulty in collecting, validating and interpreting the recorded AMU data, an audit system of the empty antimicrobial containers was preferred over treatment

diaries by Carson et al. (2008), and therefore this system was employed in the present study for the same reasons as well. The prospective design of the study and placement of receptacles for collecting antimicrobials prevented issues of recall bias, and of incomplete and unverifiable records associated with self-reported surveys and treatment diaries. The audit system was a producer and technician friendly system to determine herd-level AMU. However, it is quite likely that producers might have forgotten to put all of the empty antimicrobials in these receptacles. Still, the authors would recommend using such a system for collecting herd-level information on AMU. However, electronic recorders for recording animal level use of antimicrobials are also needed. At the animal level, electronic recording systems would promote better accuracy and traceability of treatments (González et al., 2010; Carson et al., 2008; Singer et al., 2006).

The veterinary analogue of defined-daily dose (animal defined-daily dose; ADD) holds a specific relevance in pharmaco-epidemiologic studies of drug consumption. Animal defined-daily dose corrects for differences in the therapeutic potency of active ingredients and formulations of the antimicrobial drugs (Chauvin et al., 2001). Further, the ADD considers pharmacological activity of an antimicrobial agent in exerting selection pressure applied to a dairy farm environment. The ADD has been used to describe veterinary AMU at the country level and the farm level as a unit for standardized drug utilization (Grave et al., 1999; Grave et al., 2004; Jensen et al., 2004; Pol and Ruegg, 2007; Carson et al., 2008). In the context of dairy farming in Canada, the use of ADD at farm level is a novel approach for studying on-farm AMU. Nevertheless, ADD used in the present study cannot be compared with similar units used in other studies due to variation in pharmacopoeia and body weights of farm animals assumed in the calculation protocol. Further, ADD only

considers average on-label recommended dosage for the indicated conditions and fails to consider extra-label drug use. Therefore, if the extra-label drug usage varies between geographical regions, ADD would fail to reflect this difference. Further, ADD fails to distinguish between the treatment protocol of 1-2 syringes per cow in 24 hours for clinical mastitis treatment and the protocol of 4 tubes per cow for dry cow therapy. Even though the number of syringes used per animal for dry cow therapy is much more than for clinical mastitis treatment, both treatment protocols would still constitute a single ADD. In fact, ADD is just a scaling factor and an index measure for comparing AMU between different farms (Jensen et al., 2004).

Overall ADUR was found to be positively but weakly correlated with herd-level milk production. Overall ADUR was not significantly associated with average herd SCC and herd size, barn type or geographical regions. Similarly, Zwald et al. (2004) had found no association between herd size and antimicrobial use in their study. However, the use of some specific antimicrobial drug classes correlated with herd milk production level and herd size. For example, positive correlation between herd-level milk production and ADUR of systemic third-generation cephalosporins (ceftiofur), intramammary first-generation cephalosporins (cephapirin) used for dry cow therapy, and intramammary lincosamides administered for clinical mastitis treatment was evident in the present study; however, it is highly likely that such an association is confounded by higher incidence of mastitis and (or) differences in producers' attitudes and preferences about mastitis treatment on these farms. Similarly, ADUR of systemic third-generation cephalosporins (ceftiofur) and average herd size was also correlated. Tie-stalls and free-stalls were the most common housing types on Canadian dairy farms. Overall ADUR was not associated with barn type. However, ADUR

of penicillin combinations used for intramammary clinical mastitis treatment, and systemically administered penicillins tended to be greater in tie-stall dairy herds than in free-stalls even though such differences were not significant. Incidence of clinical mastitis and distribution of mastitis causing pathogens varied by barn-types (Olde Riekerink et al., 2008) and BMSCC (Barkema et al., 1998) thereby potentially explaining heterogeneity in AMU between farms within and across regions. Interestingly, correlation between intramammary ADUR and average herd SCC was not observed in the present study. It is plausible that SCC being an intermediate variable on causal pathway between incidence of mastitis (subclinical or clinical) and intramammary ADUR does not have an independent impact on ADUR per se or the impact is negligible. Data on incidence of mastitis should therefore be collected to evaluate relationship between SCC and ADUR in antimicrobial drug utilization studies. In addition, information on herd-level management practices that could potentially impact incidence of disease and therefore ADUR should be collected as well.

Variation in the use of antimicrobials among dairy farms was evident in the present study as also observed by Pol and Ruegg (2007) and González et al. (2010). Cephalosporins, penicillins, penicillin combinations, and tetracyclines were the most commonly used antimicrobial drug classes on Canadian dairy farms whereas macrolides and fluoroquinolones were infrequently used; these observations are similar to what was found on Dutch dairy farms (MARAN, 2008). Generally speaking, ß-lactams were used on all dairy farms, and constituted the highest proportion of AMU - similar to what is found on US dairy farms (Sawant et al., 2005; Raymond et al., 2006). Within ß-lactams, cephalosporins had greater ADUR than penicillins. Within cephalosporins, third-generation

cephalosporins (ceftiofur) had greater ADUR than first-generation cephalosporins (cephapirin); Pol and Ruegg (2007) and Zwald et al. (2004) had also reported the frequent use of ceftiofur for treating various diseases on the majority of dairy farms. Ceftiofur, when administered systemically as per indicated dose and duration, does not have a withdrawal period for milk or meat (Erskine et al., 2002), and is therefore an attractive antimicrobial to use on dairy farms. The use of third and fourth-generation cephalosporins (ceftiofur in particular) tended to increase on Dutch dairy farms as well (MARAN, 2008).

Health Canada has categorized antimicrobial drug classes considering that some antimicrobial classes are considered more important in treatment of serious bacterial infections, and that development of resistance against these classes will have more serious consequences for human health (Health Canada, 2009). These categories based on importance in human medicine, are as follows: category I – very high importance (e.g. third-generation cephalosporins, penicillin combinations containing polymyxin (colistin), fluoroquinolones), category II – high importance (e.g. first-generation cephalosporins, aminoglycosides, lincosamides, macrolides, penicillins, and TMP-sulfonamide combinations), category III – medium importance (e.g. phenicols, tetracyclines, sulfonamides), and category IV – low importance (e.g. ionophores). In the present study, category II drug classes were used most on Canadian dairy farms followed by category I, category IV and category III antimicrobials.

In case of antimicrobials administered via systemic route, significant differences in ADUR of third-generation cephalosporins (ceftiofur) and tetracyclines were evident across the 4 studied regions. Dairy herds in Maritimes were lower users of systemic tetracyclines in

comparison to Alberta whereas dairy herds in Québec were lower users of third-generation cephalosporins (ceftiofur) than Ontario and Maritimes. Penicillins, novobiocin and cephalosporins (ceftiofur in particular) were antimicrobials of choice in Ontario dairy herds consistent with what was previously observed by Léger et al (2003). Differences in antimicrobial prescription policy between provinces can potentially explain geographical variation in AMU. For example, antimicrobials cannot be purchased without veterinary prescription in Québec unlike the rest of Canada where some antimicrobials can be purchased. In addition, at least one visit to the dairy farm in the preceding 12 months by the veterinarian is required in order to prescribe a veterinary compound in Québec. These regulations might influence the type of antimicrobial use in this province.

Intramammary dry cow therapy was adopted on almost all (98%) the dairy herds in this study, an observation similar to Zwald et al. (2004), Raymond et al. (2006), and Pol and Ruegg (2007). Among intramammary drugs, antimicrobials were used in greater proportion for clinical mastitis treatment as compared to dry cow therapy as also observed on US dairy farms (Pol and Ruegg, 2007) and Finnish dairy farms (FINRES – Vet, 2005 – 2006). First-generation cephalosporins (cephapirin) and penicillins (cloxacillin, and penicillin G procaine-novobiocin combination) were most commonly used for dry cow therapy, an observation similar to Raymond et al. (2006) and Pol and Ruegg (2007). Further, penicillin combinations were used in higher proportion on the majority of dairy farms relative to first-generation cephalosporins (cephapirin) and lincosamides (pirlimycin) for intramammary clinical mastitis treatment unlike the study conducted by Pol and Ruegg (2007) where first-generation cephalosporins (cephapirin) and lincosamides (pirlimycin) were the most frequently used intramammary products for clinical mastitis treatment. However, penicillin

combinations are not available as intramammary mastitis treatment preparations in lactating cows in the US. It can be concluded that ß-lactams are still the most commonly used intramammary preparations for prevention and treatment of mastitis, as observed in other studies as well (Zwald et al., 2004; Sato et al., 2005; Sawant et al., 2005; Pol and Ruegg, 2007).

Dairy herds in this study were not randomly selected. However, these herds were representative of their respective dairy herd populations in some important parameters (Reyher et al., 2011). Further, due to a lack of information on herd size in terms of calf and heifer inventories on each farm, the antimicrobial drug use rates over estimate the actual AMU on farms. Furthermore, inference from this herd-level study could not be applied at the animal level due to ecological fallacy. Also, the study excluded 3times milking per day herds that have higher milk production and potentially lower clinical mastitis incidence (Smith et al., 2002) and therefore lower drug use. In general, the AMU data can be used as a baseline to monitor temporal trends in antimicrobial drug utilization on Canadian dairy farms, and also to evaluate the impact of interventions for promoting judicious use of antimicrobials in Canadian dairy farming.

3.6 Conclusions

Variation in antimicrobial use between dairy farms within and across 4 regions was evident. Overall ADUR increased with an increasing herd-level milk production, but was not associated with average herd SCC, herd size, barn type, and geographical region. ß-lactams were the most commonly used antimicrobials on Canadian dairy farms. Among antimicrobials of very high importance in human medicine, the use of fluoroquinolones was

rare whereas third-generation cephalosporins and penicillin combinations containing colistin were used very frequently. Coordinated ongoing surveillance of antimicrobial use is needed to determine the impact of antimicrobial use on antimicrobial resistance.

3.7 References

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Table 3.1: National level estimate of antimicrobial drug use rate (ADUR; Animal defined-daily doses (ADD)/1000 cow-days) of various antimicrobial drug classes used across 89 Canadian dairy farms, 2007-2008.

			ADUR[1] percentile								
Drug Class	Herds	ADD[2] (95% CI)	ADUR	Min.	5th	10th	25th	50th	75th	90th	Max.
Cephalosporins – 1st	76 (87)	3,451 (3,320 to 3,559)	0.85	0	0	0	0.10	0.41	1.38	2.59	7.07
Cephalosporins – 3rd	80 (90)	8,949 (8,738 to 9,086)	2.20	0	0	0	0.55	1.24	2.94	5.29	7.34
Cephalosporins – All	87 (98)	12,400 (11,649 to 12,581)	3.05 [a]	0	0.27	0.45	0.99	2.70	3.74	6.06	8.94
Penicillins	85 (96)	10,421 (10,193 to 10,601)	2.56 [a]	0	0.08	0.50	1.63	2.37	3.50	4.59	7.20
All ß-lactams	89 (100)	22,821 (22,542 to 23,008)	5.62	0.45	1.41	2.26	3.36	5.01	6.63	9.33	12.87
Penicillin Combination[3]	84 (94)	8,942 (8,737 to 9,086)	2.20 [a]	0	0	0.05	0.53	1.65	2.99	3.93	19.68
Tetracyclines	57 (64)	7,445 (7,281 to 7,572)	1.83[a]	0	0	0	0	0.36	0.92	2.71	50.89
TMP-sulfa[4]	68 (76)	3,539 (3,378 to 3,611)	0.87[a]	0	0	0	0.07	0.52	1.34	2.33	3.96
Lincosamides	52 (58)	3,414 (3,261 to 3,494)	0.84	0	0	0	0	0.04	0.73	2.35	8.91
Macrolides	31 (35)	1,163 (1,048 to 1,223)	0.28	0	0	0	0	0	0.10	0.71	5.41
Phenicols	29 (33)	694 (640 to 699)	0.17	0	0	0	0	0	0.15	0.53	1.21
Aminoglycosides	10 (11)	429 (349 to 465)	0.10	0	0	0	0	0	0	0.09	1.28
Ionophores	4 (5)	318 (232 to 349)	0.07	0	0	0	0	0	0	0	3.79
Fluoroquinolones	4 (5)	11 (5 to 19)	0.003	0	0	0	0	0	0	0	0.15

Sulfonamides	2 (2)	9 (4 to 17)	0.002	0	0	0	0	0	0	0	0.08
Linco-Spectinomycin[5]	1 (1)	9,464 (9,261 to 9,611)	2.33	0	0	0	0	0	0	0	89.61
Overall	89 (100)	58,249	14.35	1.76	3.30	3.63	6.03	8.67	14.34	22.5	105.00

[a]Antimicrobial drug use rates of five most commonly used antimicrobial drug classes ($P < 0.05$).

[1]Differences in ADUR among antimicrobial drug classes at national level were determined using chi-square test ($P < 0.05$).

[2] Number of animal defined-daily doses of an antimicrobial drug class. Non-overlapping 95% confidence intervals around ADD indicate statistically significant differences in ADUR among various antimicrobial classes.

[3]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

[4]Trimethoprim – sulfadoxine combination.

[5]Lincomycin – Spectinomycin combination.

Table 3.2: Regional level estimate of antimicrobial drug use rate (ADUR; Animal Defined-Daily Doses/1000 cow-days) by drug class on 89 dairy farms in 4 regions of Canada, 2007-2008.

	Alberta (n=17)			Ontario (n=27)			Québec (n=28)			Maritimes (n=17)			
Drug Class	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	*P*-value[1]
Cephalosporins – 1st Gen.	15 (88)	0.32	0.30	21 (78)	0.93	0.42	24 (86)	0.89	0.42	16 (94)	1.30	0.82	0.14
Cephalosporins – 3rd Gen.	15 (88)	2.08	1.20	27 (100) [a]	2.97	1.94	22 (79) [a]	1.24	0.68	16 (94)	2.55	1.55	0.003
Penicillins	16 (94)	2.47	2.31	25 (93)	2.29	1.81	28 (100)	2.83	2.92	16 (94)	2.68	2.67	0.12
Penicillin Combination[2]	17 (100)	2.73	2.43	24 (89)	1.63	1.30	27 (96)	2.17	1.85	16 (94)	2.34	1.43	0.34
Tetracyclines	15 (88)[a]	3.68	1.25	15 (55)	0.68	0.36	20 (71)	0.68	0.31	7 (41)[a]	2.47	0	0.003
TMP-sulfa[3]	15 (88)	1.37	0.87	17 (63)	0.58	0.16	24 (86)	0.92	0.62	12 (71)	0.57	0.45	0.08
Lincosamides	12 (71)	1.33	0.40	16 (59)	1.19	0.07	17 (63)	0.63	0.12	7 (41)	0.08	0	0.08
Macrolides	5 (29)	0.44	0	11 (41)	0.31	0	10 (36)	0.31	0	5 (29)	0.03	0	0.72
Phenicols	9 (53)	0.22	0.10	9 (33)	0.11	0	7 (25)	0.21	0	4 (23)	0.13	0	0.22
Aminoglycosides	2 (12)	0.20	0	1 (4)	0.03	0	5 (18)	0.16	0	2 (12)	0.005	0	0.43
Ionophores	1 (6)	0.20	0	2 (8)	0.002	0	1 (4)	0.10	0	0	0	0	0.70
Fluoroquinolones	2 (12)	0.008	0	0	0	0	2 (8)	0.002	0	0	0	0	0.20
Sulfonamides	1 (6)	0.002	0	1 (4)	0.005	0	0	0	0	0	0	0	0.51

Drug Class	Alberta (n=17)			Ontario (n=27)			Québec (n=28)			Maritimes (n=17)			
	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	*P*-value[1]
Linco-Spectinomycin[4]	0	0	0	1 (4)	8.73	0	0	0	0	0	0	0	0.51
All ß-lactams	17 (100)	4.87	4.00	27 (100)	6.19	5.57	28 (100)	4.96	4.85	17 (100)	6.53	5.70	0.37
Overall	17 (100)	15.05	10.19	27 (100)	19.51	9.35	28 (100)	10.19	8.94	17 (100)	12.20	8.26	0.57

[a] Regions differed in the distribution of antimicrobial drug use rate from each other (Wilcoxon rank-sum test, $P < 0.05$).

[1]Statistical significance of variation in distribution of ADUR across 4 regions within a drug class was determined by Kruskal Wallis test ($P < 0.05$).

[2]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

[3]Trimethoprim – sulfadoxine combination.

[4]Lincomycin – Spectinomycin combination.

Table 3.3: Regional level estimates of antimicrobial drug use rate (ADUR; Animal Defined-Daily Doses/1000 cow-days) by route of administration on 89 dairy farms in 4 regions of Canada, 2007-2008.

	Alberta (n=17)			Ontario (n=27)			Québec (n=28)			Maritimes (n=17)			
Route	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	*P*-value[1]
Intramammary	17 (100) [a]	5.73	4.41	25 (93) [b]	4.26	4.19	28 (100) [c]	5.38	4.46	17 (100) [d]	5.02	3.23	0.44
Systemic	17 (100) [a]	6.47	4.29	27 (100) [b]	5.79	5.00	27 (96) [c]	4.61	3.93	17 (100) [e]	4.89	3.16	0.29
Other[2]	8 (47) [a]	2.84	0	10 (37) [b]	9.46	0	5 (18) [c]	0.19	0	6 (35) [d,e]	2.28	0	0.23

[a-e]Antimicrobial drug use rates within a column with common superscripts differ (chi-square $P < 0.05$).

[1]Statistical significance of variation in ADUR among 4 regions within a route of administration was determined by Kruskal Wallis test ($P < 0.05$).

[2]Other routes included topical, oral and intrauterine route of drug administration.

Table 3.4: National level estimate of intramammary antimicrobial drug use rate (ADUR; Animal Defined-Daily Doses/1000 cow-days) by antimicrobial drug classes administered for dry cow therapy and clinical mastitis treatment on 89 Canadian dairy farms, 2007-2008.

Drug Class	National Level ADUR[1]	Dry cow therapy					Clinical mastitis therapy				
		Herds (%)	Percentile				Herds (%)	Percentile			
			ADUR	25th	50th	75th		ADUR	25th	50th	75th
Cephalosporins – 1st Gen.	0.83	42 (47)	0.27	0	0[a]	0.27	64 (72)	0.56	0	0.26[a]	0.82
Cephalosporins – 3rd Gen.	0.09	-	-	-	-	-	28 (31)	0.09	0	0	0.07
Penicillins	1.28	83 (93)	1.28	0	1.34	1.84	-	-	-	-	-
Penicillin Combination[2]	2.20	-	-	-	-	-	84 (94)	2.20	0.53	1.65	2.99
Lincosamides	0.66	-	-	-	-	-	52 (58)	0.66	0	0.04	0.70
Macrolides	0.004	3 (3)	0.003	0	0	0	1 (1)	0.001	0	0	0
All ß-lactams	2.21	87 (98)	1.55	1.19	1.67[a]	1.94	71 (80)	0.66	0.06	0.39[a]	0.94
Overall	5.07	87(98)	1.55	1.19	1.67[a]	1.94	87 (98)	3.52	1.22	2.68[a]	4.58

[a] Antimicrobial drug use rates within a row differ from each other (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$)

[1]Combined ADUR of intramammary antimicrobials used for dry cow therapy and clinical mastitis treatment.

[2]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

Table 3.5: Regional level estimates of intramammary antimicrobial drug use rate (ADUR; Animal Defined-Daily Doses/1000 cow-days) of antimicrobial drug classes used for dry cow therapy and clinical mastitis treatment on 89 dairy farms in 4 regions of Canada, 2007-2008.

	Alberta (n=17)			Ontario (n=27)			Québec (n=28)			Maritimes (n=17)			
Drug Class[1]	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	*P*-value[2]
Dry cow therapy													
Cephalosporins – 1st Gen.	5 (29)	0.08	0	13 (48)	0.48	0	13 (46)	0.16	0	11 (65)	0.34	0.07	0.15
Penicillins	16 (94)	1.32	1.34	23 (85) [a]	1.01	0.84	28 (100) [a]	1.52	1.64	16 (94)	1.27	1.59	0.03
All ß-lactams	17 (100)	1.40	1.48	25 (93)	1.49	1.68	28 (100)	1.68	1.74	17 (100)	1.61	1.75	0.23
Clinical mastitis therapy													
Cephalosporins – 1st Gen.	12 (71) [a]	0.22	0.25	16 (59)	0.42	0.07	21 (75)	0.72	0.34	15 (88) [a]	0.95	1.12	0.04
Cephalosporins – 3rd Gen.	4 (23)	0.06	0	12 (44)	0.14	0	10 (36)	0.14	0	2 (12)	0.01	0	0.12
Lincosamides	12 (71)	1.33	0.40	16 (59)	0.53	0.04	17 (61)	0.63	0.12	7 (41)	0.08	0	0.08
Penicillin Combination[3]	17 (100)	2.70	2.43	24 (89)	1.63	1.30	27 (96)	2.17	1.85	16 (94)	2.34	1.43	0.34
All ß-lactams	12 (71)	0.28	0.32	20 (74)	0.56	0.24	23 (82)	0.86	0.47	16 (94)	0.96	0.56	0.07

[a] Regions differed in the distribution of antimicrobial drug use rate from each other (Wilcoxon rank-sum test, $P < 0.05$).

[1]Within all regions, ADUR differed among various classes used for dry cow and clinical mastitis treatment (chi-square test, $P < 0.05$). No differences between ADUR of first-generation cephalosporins and lincosamides used intramammary for clinical mastitis treatment were observed in dairy herds in Québec.

[2]Statistical significance of variation in distribution of ADUR across 4 regions within a drug class was determined by Kruskal Wallis test ($P < 0.05$).

[3]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

Table 3.6: Regional level estimates of systemic antimicrobial drug use rate (ADUR; Animal Defined-Daily Doses/1000 cow-days) of antimicrobial drug classes administered on 89 dairy farms in 4 regions of Canada, 2007-2008.

	Alberta (n=17)			Ontario (n=27)			Québec (n=28)			Maritimes (n=17)			
Drug Class[1]	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	Herds (%)	ADUR	Median	*P*-value[2]
Cephalosporins – 3rd Gen.	15 (88)	2.01	1.20	27 (100) [a]	2.83	1.93	19 (68) [a,b]	1.09	0.55	16 (94)	2.54	1.55	0.001
Penicillins	12 (71)	1.15	0.63	22 (81)	1.28	0.80	25 (89)	1.30	1.39	16 (94)	1.41	0.89	0.68
Tetracyclines	15 (88) [a]	1.25	0.72	15 (55)	0.68	0.36	20 (71)	0.68	0.31	6 (35) [a]	0.19	0	0.006
TMP-sulfa[3]	15 (88)	1.37	0.87	17 (63)	0.58	0.16	24 (86)	0.92	0.62	12 (71)	0.57	0.45	0.08
Macrolides	5 (29)	0.44	0	9 (33)	0.30	0	10 (36)	0.31	0	3 (18)	0.03	0	0.43
Phenicols	9 (53)	0.22	0.10	9 (33)	0.11	0	7 (25)	0.21	0	4 (23)	0.13	0	0.22
Aminoglycosides	0	0	0	0	0	0	1 (4)	0.07	0	0	0	0	0.53
Fluoroquinolones	2 (12)	0.008	0	0	0	0	2 (7)	0.002	0	0	0	0	0.20
All ß-lactams	17 (100)	3.17	3.12	27 (100)	4.11	3.30	27 (96)	2.39	1.75	17	3.95	2.14	0.16

[a,b] Regions with common superscripts differed in the distribution of antimicrobial drug use rate from each other (Wilcoxon rank-sum test, $P < 0.05$).

[1]Within all regions, ADUR differed among various antimicrobial classes (chi-square $P < 0.05$). However, no differences in ADUR between penicillins and tetracyclines, and between tetracyclines and TMP-sulfadoxine combinations were observed in dairy herds in Alberta.

[2]Statistical significance of variation in distribution of ADUR across 4 regions within a drug class was determined by Kruskal Wallis test ($P < 0.05$).

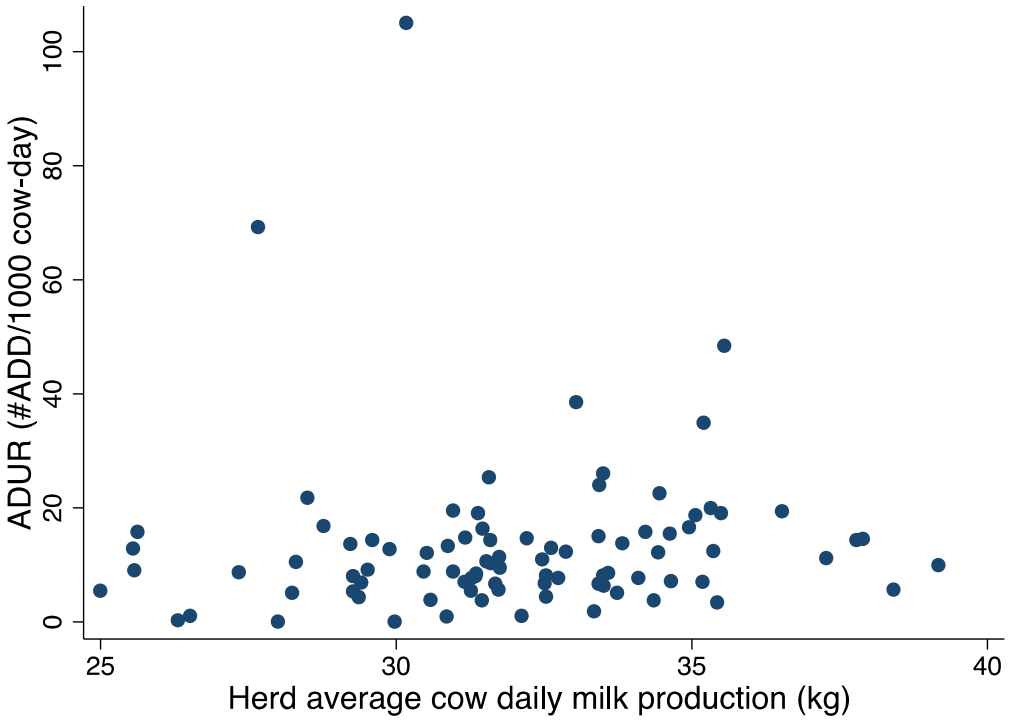
[3]Trimethoprim – sulfadoxine combination.

Table 3.7: Correlations between herd-level antimicrobial drug use rate (ADUR; Animal defined-daily doses (ADD)/1000 cow-days) and average herd milk production, average herd somatic cell count and average herd size on 89 Canadian dairy farms, 2007-2008.

	Spearman's rho (*P*-value)		
Antimicrobial drug use rate (ADD/1000 cow-days)	Milk production (kg)	Somatic cell count (,000 cells/ml)	Herd size
Overall ADUR	0.21 (*P* = 0.04)	0.07 (*P* = 0.49)	0.01 (*P* = 0.97)
Systemic cephalosporins – 3rd Gen.	0.27 (*P* = 0.01)	- 0.01 (*P* = 0.95)	0.25 (*P* = 0.01)
Systemic penicillins	0.08 (*P* = 0.44)	0.01 (*P* = 0.90)	- 0.19 (*P* = 0.07)
Systemic tetracyclines	0.09 (*P* = 0.37)	0.09 (*P* = 0.40)	0.05 (*P* = 0.59)
Systemic TMP-sulfonamide combinations	0.16 (*P* = 0.12)	0.14 (*P* = 0.18)	- 0.07 (*P* = 0.47)
Intramammary cephalosporins – 1st Gen. (dry cow therapy)	0.22 (*P* = 0.03)	- 0.03 (*P* = 0.71)	- 0.06 (*P* = 0.53)
Intramammary penicillins (dry cow therapy)	- 0.16 (*P* = 0.11)	- 0.03 (*P* = 0.77)	0.05 (*P* = 0.60)
Intramammary cephalosporins – 1st Gen. (clinical mastitis therapy)	0.04 (*P* = 0.66)	0.001 (*P* = 0.98)	- 0.09 (*P* = 0.35)
Intramammary penicillin combination (clinical mastitis therapy)[1]	0.14 (*P* = 0.16)	0.16 (*P* = 0.12)	0.05 (*P* = 0.61)
Intramammary lincosamides (clinical mastitis therapy)	0.27 (*P* = 0.01)	- 0.08 (*P* = 0.42)	- 0.01 (*P* = 0.88)

[1]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

Figure 3.1: Relationship between antimicrobial drug use rate (ADUR: Animal Defined-Daily dose (ADD)/1000 cow-days) and herd average cow daily milk production (kg) on 89 Canadian dairy farms in 6 provinces, 2007-2008



Chapter Four: Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms

4.1 Abstract

Various national and international bodies are monitoring antimicrobial resistance (AMR) in bacteria. The present study determined prevalence of AMR in common mastitis pathogens *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) (n=1810), *Escherichia coli* (n=394) and *Klebsiella* species (n=139), including extended-spectrum ß-lactamase (ESBL) producing *E. coli* and *Klebsiella* species, isolated from milk samples on 89 dairy farms in six Canadian provinces. Minimum inhibitory concentrations were determined using Sensititre® bovine mastitis plate and NARMS Gram-negative panel. Denim blue chromogenic agar and real-time PCR was used to screen and confirm MRSA, respectively.

Prevalence of AMR in *S. aureus* isolates was 20.4% (95% CI: 17.2 to 24.0%; range: 0% for cephalothin and oxacillin – 8.8% for penicillin) and 15% of isolates were multi-drug resistant (MDR). One MRSA isolate was confirmed (prevalence: 0.05%). Prevalence of AMR in *E. coli* and *Klebsiella* species isolates was 17.7% (95% CI: 14.1 to 21.9%; range: 0% for ceftriaxone and ciprofloxacin – 14.8% for tetracycline) and 36.6% (95% CI: 28.6 to 45.2%; range: 0% for amikacin, ceftiofur, ciprofloxacin and nalidixic acid – 18.6% for tetracycline), respectively. Further, 62.8 and 55% of the resistant *E. coli* and *Klebsiella* species isolates were MDR, respectively. Resistance to > 5 and > 2 antimicrobials was most common in *E. coli* and *Klebsiella* species isolates, respectively. No ESBL producers were found.

In conclusion, prevalence of AMR in bovine mastitis pathogens was low. Most Gram-negative udder pathogens were MDR. MRSA was rarely found and ESBL *E. coli* and *Klebsiella* species isolates were absent in Canadian milk samples.

4.2 Introduction

In the context of food-animal production systems, mastitis is the leading cause of antimicrobial use on dairy farms (Saini et al., 2011a). It is the most common and most economically significant disease afflicting the dairy industry, which is ranked third in terms of value after grains and red meat in Canadian agriculture economy (Agri-Food Canada, 2007). A variety of bacteria can be isolated from bovine mastitis cases. The most frequently isolated major pathogens are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*, and enteric bacteria such as *Escherichia coli* and *Klebsiella* species. *S. aureus* and *E. coli* are the most common causes of contagious and environmental clinical mastitis, respectively (Barkema et al., 1998; Olde Riekerink et al., 2008), while *Klebsiella* is an emerging pathogen with rising incidence in North America (Zadoks and Munoz, 2007). *S. aureus*, *E. coli*, *S. uberis* and coagulase-negative staphylococci were the most commonly isolated bovine clinical mastitis pathogens in Canada, while *Klebsiella* species was the most common udder pathogen in free-stall dairy cattle herds in Western Canada (Olde Riekerink et al., 2008).

Antimicrobial therapy is the preferred way for mastitis prevention and control. Unfortunately, despite best possible antimicrobial treatments, bacteriological cure failures are common, especially for *S. aureus* mastitis, and antimicrobial resistance (AMR) is one of the reasons for low cure rates (Barkema et al., 2006). Additionally, AMR in bacteria is a public health hazard, and antimicrobial use is considered as a potentially important driver of AMR (White and McDermott, 2001). For example, cloxacillin, an antimicrobial similar

to methicillin/oxacillin is extensively used for dry cow therapy (Saini et al., 2011a), and it is hypothesized that cloxacillin use may potentially select for methicillin-resistance in *S. aureus*. In fact, methicillin-resistant *S. aureus* (MRSA) have been isolated from mastitis milk samples in dairy cattle (Huber et al., 2010; Vanderhaeghen et al., 2010) and have shown genetic relatedness to human MRSA strains (Lee, 2003), thereby suggesting an inter-species mode of transmission (Moon et al., 2007). Further, livestock can be reservoirs of resistance genes such as those associated with the production of extended-spectrum ß-lactamases (ESBL) in Enterobacteriaceae that could also be transferred to humans (Fey et al., 2000; Schwarz et al., 2001). In addition to expanded-spectrum cephalosporins (examples: ceftriaxone, ceftazidime, cefotaxime, and ceftiofur), ESBL producers frequently carry resistance determinants that confer resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole combinations. Ceftiofur is commonly used to treat dairy cattle including systemic treatment of coliform mastitis (Wagner and Erskine, 2006), and ESBL *E. coli* and *Klebsiella* species isolates have been isolated from milk from dairy cattle (MARAN, 2008; Hammad et al., 2008) and from milk filters on a dairy farm (Dolejska et al., 2011). In general, emergence and transfer of AMR bacteria/genetic determinants from animals to human populations via food chain is a growing concern (Piddock, 1996; Welton et al., 1998), and therefore, assessing the prevalence status of these multiple resistant pathogens such as MRSA, and ESBL *E. coli* and *Klebsiella* species in foods and food animals has clinical and public health significance. In the context of dairy farming, even though most of the milk produced is pasteurized, unpasteurized milk is still consumed by dairy producers and their families, and raw milk cheese can be sold to consumers; disease outbreaks have been linked to consumption of raw milk (Oliver et al., 2004). Further, effluents from farms containing drug residues and resistant bacteria can

enter the aquatic and terrestrial ecosystems to create environmental reservoirs of drug resistant bacteria (Chee-Sanford et al., 2001). It is therefore important to determine AMR in food animal pathogens at the pre- and post-harvest stages of food production and monitor AMR profile patterns in them over years for food safety, animal health and public health aspect.

Many countries have implemented systems for surveillance of AMR in bacterial pathogens (CIPARS, 2007; DANMAP, 2009; MARAN, 2008). There is a national recognition of the value of this type of information in Canada (Health Canada, 1997). The leading body in Canada for data collection on AMR in bacterial isolates from chicken, swine and beef cattle meat is the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (2007). Unfortunately, it does not collect information on AMR profiles of common mastitis pathogens at the retail or farm level. Hence, there is lack of information on prevalence of AMR in mastitis pathogens in milk in Canada. Some of the previous studies conducted to determine AMR profiles of bovine mastitis pathogens in Canada included isolates from clinical mastitis cases submitted to diagnostic laboratories; disk diffusion test method involving different antimicrobials than being used presently was commonly employed in these studies (Hariharan et al., 1974; MacDonald et al., 1973; Prescott, 2006). Sabour et al. (2004) assessed the antimicrobial susceptibility of *S. aureus* isolates (n=212) collected from clinical mastitis cases in 58 dairy herds in provinces of Ontario, Québec and Prince Edward Island. However, none of these studies collected information on the prevalence of MRSA, ESBL *E. coli* and *Klebsiella* species in milk samples and also did not collect isolates from subclinical mastitis cases or intramammary infection (IMI). Therefore, epidemiological differences in the sources of isolates resulted in

a biased assessment of AMR situation in bovine mastitis pathogens in Canada. Consequently, the importance of information on AMR profile patterns on Canadian dairy farms had prompted CIPARS to collaborate with various universities so to assess antimicrobial use and resistance in Canadian dairy cattle (CIPARS, 2007).

The objectives of the present study were to determine a) antimicrobial resistance profile patterns of *S. aureus, E. coli*, and *Klebsiella* species pathogens isolated from subclinical and clinical mastitis cases, and IMI and b) prevalence of MRSA, ESBL *E. coli* and *Klebsiella* species in milk samples from Canadian dairy herds.

4.3 Materials and Methods

4.3.1 Dairy Cattle Herd Selection

Milk samples were collected from the National Cohort of Dairy Farms of the Canadian Bovine Mastitis Research Network (CBMRN), which consisted of 91 commercial dairy farms located in 4 regions across Canada (Alberta, Ontario , Québec and the Maritime Provinces [Prince Edward Island, Nova Scotia, and New Brunswick]). Herd selection criteria for the present study have been described by Reyher et al. (2011). In short, dairy herds were selected to replicate the provincial proportion of free-stall systems to within 15 percentage points and to be uniformly distributed among three strata of the most recent 12-month bulk tank somatic cell count (SCC) (≤ 150,000 cells/mL, > 150,000 and ≤ 300,000 cells/mL, > 300,000 cells/mL); herds with a 3 times daily milking schedule and herds with less than 80% (or less than 15) Holstein-Friesian lactating and dry cows at the time of enrolment were excluded. Further, eligible dairy herds must have been participating in Dairy Herd Improvement data collection program. Eligible dairy farms were identified and

contacted by the regional center coordinators. Written consent to participate in the research cohort was obtained. Two dairy herds dropped out of the study at the very beginning. Average herd size of the 89 participating dairy herds was 84 cows (median: 66, range 33 – 297). Average herd milk production ranged from 25 to 39kg (mean and median: 32kg). Average herd SCC ranged from 91,000 – 500,000 cells/mL (arithmetic mean: 230,000 cells/mL; median: 220,000 cells/mL). Sixty-one, 33, 5 and 1% of these dairy herds housed lactating cows in tie-stalls, free-stalls, on a bedding pack in a free-stall and mixed barn type, respectively. In each coordinating center, an investigator and technicians were responsible for the data collection activities related to farms located in that center's area.

4.3.2 Sampling and Bacterial Culturing

Three different sets of milk samples were collected (Reyher et al., 2011). The first set included milk samples from clinical mastitis cases. All producer-diagnosed clinical mastitis cases were sampled (M1) and then re-sampled twice at two-week intervals (M2 and M3). These samples included only quarter milk samples.

The second set was from milk samples from nonclinical lactating cows; a sub-sample of 15 fresh and lactating cows was selected per farm. They were aseptically sampled and re-sampled once every 3 weeks for a total of three samplings in the winter of 2007 (L1, L2 and L3); another sub-sample of 15 lactating cows was sampled once a week for 7 weeks in the summer of 2007 (L1 – L7). Quarter and composite milk samples were collected. The 2008 data collection was similar to that of 2007 with the exception that both summer and winter samplings were done 3 times at 3-week intervals and that fresh cows were not included into the lactating cow sub-sample.

The third set of milk samples were collected from another selected group of 15 cows that were expected to remain in the herd until at least 2 weeks after calving. A sub-sample was aseptically sampled before dry-off (DC1 and DC2) and after calving (FC1 and FC2) in 2007 and this continued in 2008 as well. Quarter and composite milk samples were collected. Samples were frozen at -20^0C and shipped to the regional CBMRN laboratory where bacterial culturing and identification of the milk samples was done as per National Mastitis Council guidelines (Hogan et al., 1999). Thereafter, samples were preserved with bronopol and shipped to the University of Prince Edward Island for SCC determination.

Isolates of growth from all culture-positive quarters that were considered significant were conserved (NCDF, 2009) (for details on conservation). Because multiple isolates could be coming from a single cow, it was decided to include only one isolate per quarter. Clinical mastitis was defined as an inflammation of the udder leading to occurrence of flakes, clots or other gross alterations in milk. Subclinical mastitis was defined as SCC >200,000 cells/mL from a cow without clinical signs of mastitis, whereas IMI was defined as a culture-positive sample (Reyher et al., 2011).

4.3.3 Antimicrobials

Minimum inhibitory concentrations (MIC) of these isolates were determined using the Sensititre® microdilution system (TREK Diagnostic Systems Inc., Cleveland, Ohio). Sensititre® Mastitis plate format CMV1AMAF, and Sensititre® NARMS Gram-negative plate format CMV1AGNF were used for the Gram-positive and Gram-negative organisms, respectively. Bovine mastitis plate contains antimicrobials that are commonly used for

mastitis prevention and control, including ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, penicillin-novobiocin combination, pirlimycin, sulfadimethoxine, and tetracycline. NARMS Gram-negative plate includes the following antimicrobials: amikacin, ampicillin, amoxicillin-clavulanic acid combination (amoxicillin-CLA combination), ceftriaxone, chloramphenicol, ciprofloxacin, trimethoprim (TMP)-sulfamethoxazole combination, cefoxitin, gentamicin, kanamycin, nalidixic acid, sulfisoxazole, streptomycin, tetracycline, and ceftiofur.

4.3.4 Sensititre® System MIC Method

Antimicrobial susceptibility testing was done as per manufacturer's instructions (TREK Diagnostic Systems Inc., Cleveland, Ohio). Thereafter, 10µL of the Sensititre® Mueller-Hinton broth inoculated with *S. aureus* suspension was pipetted onto denim blue agar plates (Chromogenic MRSA Screening Agar, Oxoid, Canada), and streaked for individual colonies for detection of methicillin-resistance. Concurrently, a plate of Sensititre® Mueller-Hinton agar containing 0.25 µg/mL of penicillin G was also inoculated for detecting penicillin resistance. Denim blue agar plates, and Mueller-Hinton agar plates containing 0.25 µg/mL of penicillin G were incubated at 37± 2^0C for 18 and 48 hours, respectively. Minimum inhibitory concentrations of various antimicrobial-isolate combinations were determined using the Sensititre Auto Reader™. In case of sulfadimethoxine, the MIC results were determined manually, as advised by Trek diagnostics. Discrete denim blue colonies against white background were presumptive positive for MRSA. An MRSA-positive isolate was confirmed to be *S. aureus* using a Pastaurex-Plus latex agglutination test (Oxoid). Subsequently, genotypic confirmation of the presence of *mecA* gene for methicillin-resistance was done using a real-time PCR assay

(Paula et al., 2005). Growth on Mueller-Hinton agar plates containing 0.25 µg/mL of penicillin G indicated penicillin resistance in *S. aureus*. Further, *E. coli* and *Klebsiella* species isolates were screened for ESBL production based on ceftriaxone MIC of ≥ 2 µg/mL as recommended by CLSI (2008). Later, a Sensititre® ESBL confirm plate was to be used on suspect isolates for confirmation of ESBL production, and finally, all ESBL isolates were to be confirmed as *E. coli* and/or *Klebsiella* species using a Sensititre® Gram-negative identification plate. Following reference strains were included in the study: *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. Isolates of interest in this study i.e. *S. aureus, E. coli*, and *Klebsiella* species were stored in skim milk in a commercial freezer at -20^0C.

4.3.5 Statistical Analyses

Data were checked for unlikely observations; however, no unlikely values were excluded. Minimum inhibitory concentrations were determined; MIC was defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate. In case of antimicrobial combinations such as penicillin-novobiocin, amoxicillin-CLA and TMP-sulfamethoxazole, the MIC of the first agent (penicillin, amoxicillin, TMP) was reported as the MIC for the combination. MIC_{50} and MIC_{90} values were also determined for various isolate-antimicrobial combinations. The MIC_{50} and MIC_{90} value represented the minimum concentration of an antimicrobial, which inhibited the visible growth of ≥ 50% and ≥ 90% of the isolates, respectively. Thereafter, the isolates were categorized as sensitive, intermediate and resistant on the basis of CLSI based MIC breakpoints (2008). Intermediate isolates were combined with resistant isolates for the sake of statistical analysis. Proportions of isolates that were resistant to at least one antimicrobial were determined, and

95% exact binomial confidence intervals were calculated. Proportions of multi-drug resistant (MDR) isolates were also determined. Multi-drug resistance was defined as resistance to two or more antimicrobials. Prevalence of MRSA, ESBL *E. coli* and *Klebsiella* species isolates was determined as well.

Univariate associations were determined between antimicrobial susceptibility results (sensitive or resistant) and source of isolates (IMI, subclinical mastitis, clinical mastitis) using chi-square tests, or Fisher's exact test (in case of expected cell frequencies < 5). Data analyses were performed using Intercooled Stata®11.1 (Intercooled Stata for Macintosh, version 11.1, Stata Corporation, College Station, TX). In all instances, a *P*-value < 0.05 was considered as statistically significant.

4.4 Results

4.4.1 Staphylococcus aureus

MIC values were determined for 562 isolates that came from 562 quarters of 462 cows on 79 dairy farms. Except for ceftiofur and sulfadimethoxine, the MIC_{90} values were less than or equal to the lowest antimicrobial concentration tested. Notably, MIC_{90} value of sulfadimethoxine was 3 dilutions higher than its MIC_{50} value, whereas no difference in MIC_{50} and MIC_{90} value was evident for ceftiofur (Table 4.1). Overall, prevalence of AMR was 20.4% (95% CI: 17.2 to 24.0%). Resistance proportions ranged from 0% for cephalothin and oxacillin to 8.8% for penicillin (Table 4.2). There were no differences in resistance proportions between isolates from IMI, subclinical and clinical mastitis cases.

Among the 114 resistant *S. aureus* isolates, 17 (15.0%) isolates were found to be MDR. Resistance to 2 antimicrobials was most common among MDR isolates. Ampicillin–penicillin, and penicillin–sulfadimethoxine resistance pattern was found in 9 and 1 isolate, respectively. One isolate exhibited ampicillin–penicillin–sulfadimethoxine resistance pattern whereas two isolates exhibited ampicillin–penicillin–ceftiofur resistance pattern. Further, two isolates exhibited erythromycin–penicillin-novobiocin combination–pirlimycin resistance pattern whereas one isolate exhibited erythromycin– penicillin-novobiocin combination–pirlimycin–sulfadimethoxine–tetracycline resistance pattern. Notably, one isolate also exhibited erythromycin–pirlimycin resistance pattern (macrolide–lincosamide resistance).

A total of 1802 *S. aureus* isolates were screened for methicillin-resistance using Chromogenic MRSA Screening Agar, and penicillin resistance using Mueller-Hinton agar plates containing penicillin G. There was 1 confirmed case of MRSA (prevalence: 0.05%). This isolate was not found among the original 562 isolates that were used for MIC testing, but among the additional 1240 isolates that were screened for methicillin-resistance. The MRSA isolate was sensitive to ceftiofur (MIC $\leq$ 2 µg/mL), cephalothin (MIC $\leq$ 2 µg/mL), erythromycin (MIC $\leq$ 0.25 µg/mL), oxacillin (MIC $\leq$ 2 µg/mL), penicillin-novobiocin (MIC $\leq$ 1/2 µg/mL), and pirlimycin (MIC $\leq$ 0.5 µg/mL), and resistant to ampicillin (MIC = 8 µg/mL), penicillin (MIC = 8 µg/mL), sulfadimethoxine (MIC > 256 µg/mL) and tetracycline (MIC > 8 µg/mL). The prevalence of penicillin resistance determined using Mueller-Hinton agar plates containing 0.25 µg/mL of penicillin G was 6.9% (125/1802 isolates).

4.4.2 Escherichia coli

MIC values were determined for 394 isolates that came from 394 quarters of 353 cows on 76 dairy farms. Majority of MIC values were below the resistance breakpoints. Notably, MIC_{90} value of tetracycline was 3 dilutions higher than its MIC_{50} value (Table 4.3). Overall, prevalence of AMR was 17.7% (95% CI: 14.1 to 21.9%). Resistance proportions ranged from 0% for ceftriaxone and ciprofloxacin to 14.8% for tetracycline (Table 4.4). There were no differences in resistance proportions between isolates from IMI, subclinical and clinical mastitis cases. Further, there were also no differences in MIC_{50} and MIC_{90} values in majority of antimicrobials. However, MIC_{90} value of tetracycline in subclinical and clinical mastitis isolates was 3 dilutions higher than their MIC_{50} value. Furthermore, MIC_{90} value of sulfisoxazole in subclinical mastitis isolates was more than 4 dilutions higher than the MIC_{50} value, and MIC_{90} value of ampicillin in clinical mastitis isolates was 3 dilutions higher than the MIC_{50} value. Five isolates were flagged as ESBL suspects due to reduced susceptibility to ceftriaxone (MIC $\geq$ 2 µg/mL). None of them was confirmed to be an ESBL producer based on Sensititre® ESBL confirm plate.

Of the 70 resistant *E. coli* isolates, 44 (62.8%) isolates were found to be MDR (Table 4.7). Most commonly, 7 isolates were found resistant to 6 antimicrobials (ampicillin, kanamycin, streptomycin, sulfisoxazole, TMP-sulfamethoxazole combination, and tetracycline), and one isolate was found resistant to 10 antimicrobials (ampicillin, amoxicillin-CLA combination, chloramphenicol, ceftiofur, cefoxitin, kanamycin, streptomycin, sulfisoxazole, TMP-sulfamethoxazole combination, and tetracycline).

4.4.3 Klebsiella species

MIC values were determined for 139 isolates that came from 139 quarters of 114 cows on 37 dairy farms. Majority of MIC values were well below the resistance breakpoints. MIC_{90} values of sulfisoxazole and tetracycline were 4 and 3 dilutions higher than MIC_{50} values, respectively (Table 4.5). Due to intrinsic resistance to ampicillin in *Klebsiella* species isolates, ampicillin resistance was not accounted for while determining overall prevalence of AMR. Fifty-one isolates or 36.6% (95% CI: 28.6 to 45.2%) were resistant to one or more antimicrobials. Resistance proportions ranged from of 0% for amikacin, ceftiofur, ceftriaxone, ciprofloxacin and nalidixic acid to 18.6% for tetracycline (Table 4.6). There were no differences in resistance proportions between isolates from IMI, subclinical and clinical mastitis cases. However, the MIC_{90} value of amoxicillin-CLA combination in subclinical mastitis isolates was more than 4 dilutions higher than its MIC_{50} value. One isolate was flagged as suspect ESBL due to reduced susceptibility to ceftriaxone (MIC $\geq$ 2 µg/mL). However, it was not confirmed to be an ESBL producer based on Sensititre® ESBL confirm plate.

Of the 51 resistant *Klebsiella* isolates, 28 (55.0%) isolates were found to be MDR (Table 4.7). Two isolates were found resistant to 7 antimicrobials (chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, TMP-sulfamethoxazole combination, and tetracycline). Streptomycin–tetracycline, amoxicillin-CLA combination–cefoxitin, and streptomycin–sulfisoxazole resistance patterns were found in 8, 7 and 6 isolates, respectively.

4.5 Discussion

The study was designed to account for potential variation in AMR prevalence due to geographical and epidemiological differences. This is the first time that a Canada-wide study to determine AMR in common mastitis pathogens, and prevalence of MRSA, ESBL *E. coli* and *Klebsiella* species isolated from milk samples in dairy cattle has been conducted.

Antimicrobial susceptibility testing was done using the Sensititre® MIC system. Sensititre® MIC and Kirby–Bauer disk diffusion test method have recently been validated for common bovine mastitis pathogens and were found to have a moderate to high diagnostic accuracy, and very good essential and categorical agreement for most udder pathogen-antimicrobial combinations (Saini et al., 2011b). Sensititre® bovine mastitis MIC panel and NARMS Gram-negative MIC panel included antimicrobials that are most frequently used against Gram-positive and Gram-negative udder pathogens in a clinical setting or as representative of their antimicrobial drug classes. Categorization of isolates (sensitive, intermediate, resistant) was done mainly on the basis of interpretive criteria developed for human medicine, because interpretive criteria for bovine mastitis pathogens have been developed only for pirlimycin, ceftiofur and penicillin-novobiocin combination (Watts and Yancey, 1994). Further, the use of chromogenic agar is becoming common in diagnostic microbiology for fast and accurate detection of methicillin-resistance in *S. aureus*. Denim blue agar (Chromogenic MRSA Screening Agar) contains cefoxitin that renders it selective to MRSA**.** In the present study, the MRSA isolate was confirmed for the presence of low-affinity penicillin binding protein PBP2´ using latex agglutination test. Acquisition of

PBP2´ is encoded by *mecA* gene, which confers resistance to methicillin in *S. aureus* (Cattoir and Leclercq, 2010). Further, this MRSA isolate was positive on two real-time PCR assays for detecting *femA* gene (factor essential for methicillin-resistance) and *mecA* gene in *S. aureus* (Paule et al., 2005).

Isolates were collected in a similar manner on the basis of one isolate per quarter so as to ensure statistical independence. Collection of isolates from IMI, subclinical and clinical mastitis cases also helped to test the hypothesis that AMR proportion estimates differed between sources of isolates. However, such differences were not evident in the present study; a similar observation was made by Botrel et al. (2010). Further, MIC_{50} and MIC_{90} values were determined to assess bimodal or trimodal MIC distributions. Differences of several dilution steps between MIC_{50} and MIC_{90} values could point towards the presence of two or more subpopulations of bacteria. Except for sulfadimethoxine, no such differences were observed for *S. aureus* in the present study. However, in the case of *E. coli* isolated from subclinical mastitis cases, MIC_{90} value of sulfisoxazole was more than 4 serial dilutions higher than the MIC_{50} value, thereby potentially indicating the presence of different subpopulations. Similar differences were observed for *Klebsiella* species isolates as well. Such differences were also observed in case of tetracycline in Gram-negative udder pathogens. Further, MDR was defined as phenotypic resistance to two or more drugs either belonging to the same or different antimicrobial drug classes. For certain drug classes such as fluoroquinolones, resistance to different antimicrobials belonging to a drug class is mediated by a common resistance mechanism. In such cases AMR is a class effect, and resistance to a class representative can be reasonably generalized to remaining antimicrobials in that drug class. However, in ß-lactams and aminoglycosides, single class

representatives cannot be defined due to the presence of potentially diverse resistance mechanisms. AMR in such cases is not a class effect. Therefore, there is no universally accepted definition of MDR (Schwarz et al., 2010).

While comparing AMR proportions and MIC_{90} values between studies, differences in antimicrobial susceptibility testing methods, interpretive criteria, source of isolates, sampling strategy, bacteriologic culturing and identification methods, and regional differences in pathogen populations should be borne in mind (Aarestrup and Jensen, 1998; Erskine et al., 2002). Most studies have determined MIC values and/or resistance proportion estimates in (sub) clinical *S. aureus* isolates (n=76 to 2132), *E. coli* (n=93 to 1939), and *Klebsiella* species (n=215 to 637) using broth microdilution test method (FINRES-Vet, 2007; Klement et al., 2005; MARAN, 2008; Oliveira et al., 2000; Sabour et al., 2004; Salmon et al., 1998; Srinivasan et al., 2007; Welton et al., 1998), agar dilution (Klement et al., 2005; MARAN, 2008), agar disk diffusion test method (Erskine et al., 2002; Gentilini et al., 2000; Güler et al., 2005; Klement et al., 2005; Lanz et al., 2003; Makovec and Ruegg, 2003; Owens and Watts, 1988; Pengov and Ceru, 2003) or E-test (Gentilini et al., 2000; Pengov and Ceru, 2003). The majority of these studies used CLSI based clinical breakpoints for AMR profiling. However, epidemiological cut-off values were used to categorize isolates in a Finnish and Norwegian study (FINRES-Vet, 2007; NORM/NORM-VET, 2009). The comparisons with the latter studies were made after adjusting for differences in breakpoints.

Among various antimicrobials tested, resistance of *S. aureus* to penicillin was most common, as also reported in other studies (Erskine et al., 2002; FINRES-Vet, 2007;

Gentilini et al., 2000; Güler et al., 2005; Klement et al., 2005; MARAN, 2008; Makovec and Ruegg, 2003; Owens and Watts, 1988; Pengov and Ceru, 2003; Sabour et al., 2004; Vintov et al., 2003). However, in our Canadian isolates, MIC_{90} values and resistance proportions of most antimicrobials were lower than in most studies. For example, ampicillin, penicillin, erythromycin and tetracycline resistance proportions ranged from 34.9 to 63.3% (Güler et al., 2005; Makovec and Ruegg, 2003), 9.9 to 63.3% (Güler et al., 2005; Makovec and Ruegg, 2003), 0 to 11.6% (FINRES-Vet, 2007; Gentilini et al., 2000), and 5.6 to 27.9% (Güler et al., 2005; Vintov et al., 2003) respectively, across studies and they were higher than in our Canadian study. Resistance to oxacillin was absent as also observed in many studies (Güler et al., 2005; Klement et al., 2005; Pengov and Ceru, 2003; Watts and Salmon, 1997). However, some studies observed higher resistance to oxacillin than our study (0.6% (Erskine et al., 2002), 0.9% (Sabour et al., 2004), 1.0% (MARAN, 2008), 1.8% (Makovec and Ruegg, 2003), 11.8% (FINRES-Vet, 2007)). Prevalence of MRSA in our Canadian isolates was close to zero (0.05%) and lower than that reported from European countries such as Hungary (7.2%), Belgium (9.3%) and Turkey (17.2%) (Juhász-Kasazanyitzky et al., 2007; Türkyilmaz et al., 2009; Vanderhaeghen et al., 2010).

No *S. aureus* isolates were found resistant to cephalothin and similar results were observed in various studies (FINRES-Vet, 2007; Klement et al., 2005; Sabour et al., 2004; Watts and Salmon, 1997). Cephalothin is used as a class representative of first-generation cephalosporins except cefazolin in antimicrobial susceptibility testing (CLSI, 2008). Cephapirin, a first-generation cephalosporin is commonly used for dry cow therapy and intramammary clinical mastitis treatment, whereas ceftiofur hydrochloride, a third-generation cephalosporin is used for intramammary clinical mastitis treatment. Cephalothin

resistance (0%) in our Canadian isolates was lower than in many studies (0.1%, 0.2%, 1.0%) (Erskine et al., 2002; MARAN, 2008; Makovec and Ruegg, 2003). Ceftiofur resistance (0.3%) in our study was similar to that in the US studies (0.1%, 0.2%) and higher than in a Canadian and New Zealand study where no resistant isolates were observed (Erskine et al., 2002; Makovec and Ruegg, 2003; Sabour et al., 2004; Salmon et al., 1998). Generally speaking, resistance to ß-lactams (penicillins and cephalosporins) was low in *S. aureus*, despite the fact that ß-lactams are most commonly used for mastitis treatment and control in dairy cattle (Saini et al., 2011a). This suggests that most ß-lactams are still effective for treatment and control of bovine mastitis despite being extensively used in the past.

Resistance to pirlimycin and penicillin-novobiocin combination was rare as also reported in many studies (Erskine et al., 2002; FINRES-Vet, 2007; Gentilini et al., 2000; McDonald et al., 1973; Makovec and Ruegg, 2003; Sabour et al., 2004). Pirlimycin, a lincosamide antimicrobial is indicated for treatment of clinical and subclinical mastitis in lactating dairy cattle, whereas penicillin-novobiocin combination is indicated for treatment and prevention of mastitis in dry cows. Intramammary pirlimycin and penicillin-novobiocin combination is proven to be effective against Gram-positive bovine mastitis pathogens (Oliver et al., 2004). Interestingly, high levels of resistance to sulfonamides in *S. aureus* were observed (7.0%). Despite the rare use of sulfonamides for mastitis therapy in dairy cattle presently, they were very commonly used in the past. Therefore, present levels of resistance might potentially be attributed to higher usage in the past.

Multidrug resistance was uncommon in *S. aureus* isolates. Among MDR isolates, resistance to 2 antimicrobials, i.e. ampicillin and penicillin, was most common. Production of ß-lactamases is a primary reason for resistance to ß-lactams (Fluit and Visser, 2001). Aminobenzyl penicillins such as ampicillin are also susceptible to *S. aureus* ß-lactamases (Prescott et al., 1984). In comparison to the study by Güler et al. (2005), ampicillin-penicillin resistance pattern was seen in fewer isolates in the present study. Penicillin-sulfadimethoxine, and erythromycin-pirlimycin (ML) resistance pattern was observed in one (0.2%) isolate each. Sabour et al. (2004) had also reported the similar resistance patterns in 4 (1.9%) and 2 (0.9%) isolates, respectively in their Canadian study.

In case of *E. coli*, resistance to tetracyclines was most common followed by sulfonamides, ampicillin, TMP-sulfonamide combination, and aminoglycosides in declining order. In general, widespread acquired resistance to tetracyclines could be due to extensive use over many years. For example, tetracyclines were the most commonly prescribed antimicrobials administered for parenteral treatment of clinical mastitis in lactating cows (Sundolf and Miller, 1995). Sulfonamides are also occasionally used to treat septicemia caused by coliform mastitis in dairy cattle (Erskine et al., 2002). However, tetracycline (14.8%) and sulfisoxazole (9.2%) resistance proportions in *E. coli* were lower than in most studies. For example, tetracycline and sulfisoxazole resistance proportions ranged from 16.2 to 37.4% (MARAN, 2008; Makovec and Ruegg, 2003) and 16.3 to 34.1% (Makovec and Ruegg, 2003; Srinivasan et al., 2007) respectively, across studies.

The resistance to TMP-sulfamethoxazole combination was low (5.8%), potentially due to synergistic bactericidal activity of TMP and sulfonamides in a combination. MIC_{90} value of

TMP-sulfamethoxazole combination was lower (0.25 µg/mL) than in other studies where it ranged from 2 to 16 µg/mL (Klement et al., 2005; Lehtolainen et al., 2003). However, resistance proportion estimates were higher (5.8%) in comparison to many studies (2-4%) (Botrel et al., 2010; Erskine et al., 2002; Lehtolainen et al., 2003; Makovec and Ruegg, 2003).

Among aminoglycosides, resistance to streptomycin and kanamycin was more common than to amikacin and gentamicin. Amikacin, gentamicin, kanamycin and streptomycin have declining order of potency, spectrum of activity and stability to enzymes from plasmid-mediated resistance (Dolwing, 2006). Relatively low levels of aminoglycoside resistance in coliforms could be due to low usage in food animals, because of persistence of drug residues in meat. In general, resistance to amikacin (0.3%) and gentamicin (0.2%) in coliforms was rare as also observed in most studies (gentamicin resistance prevalence: 2, 0, 2.6, 1.0, 0, 0, and 0%) (Erskine et al., 2002; FINRES-Vet, 2007; Klement et al., 2005; Lanz et al., 2003; Lehtolainen et al., 2003; MARAN, 2008; NORM/NORM-VET, 2009).

In coliform mastitis cases with signs of systemic illness, ceftiofur, ampicillin and amoxicillin can be used for systemic treatment in lactating dairy cattle (Wagner and Erskine, 2006). Fewer *E. coli* isolates were resistant to ampicillin (8.2%) than in most studies (9.7 to 98.4%) (Botrel et al., 2010; Srinivasan et al., 2007). Notably, higher MIC_{90} value (64 µg/mL) was reported in the Dutch and Norwegian AMR surveillance studies (MARAN, 2008; NORM/NORM-VET, 2009) compared to this study (8.0 µg/ml). The combination of amoxicillin and clavulanic acid, a ß-lactamase inhibitor, has increased effectiveness against Enterobacteriaceae, especially *E. coli*. In this study, 1.7% of *E. coli*

isolates were resistant, which is similar to other studies 0 to 3% (Lanz et al., 2003; MARAN, 2008). In general, resistance to amoxicillin-CLA combination is uncommon in *E. coli* isolates but more common in *Klebsiella* sp. isolates (5.5%).

Chloramphenicol is banned from use in food animals in many parts of the world, however florfenicol, a chloramphenicol analogue is used. Chloramphenicol is used as an indicator to test for susceptibility to florfenicol. Therefore, chloramphenicol resistance in *E. coli* and *Klebsiella* species may reflect the use of florfenicol as well as persistence of acquired resistance when chloramphenicol was still used.

Cefoxitin, ceftriaxone and ceftiofur have high antibacterial activity against most Enterobacteriaceae and are broadly resistant to ß-lactamases. Resistance to ceftiofur (0.8%) and cefoxitin (2.5%) were uncommon and ceftriaxone was absent in our Canadian isolates. Reduced susceptibility to ceftriaxone (MIC $\geq$ 2 µg/mL) is an indicator of ESBL production in Enterobacteriaceae. However, no ESBL producing *E. coli* isolates were observed as also reported in other studies (FINRES-Vet, 2007; NORM/NORM-VET, 2009). In general, ESBL *E. coli* isolates from animals are uncommon but have been isolated from rectal/cloacal swabs in pigs, chickens, and cattle in descending order (Dolejska et al., 2011; Horton et al., 2011) whereas reports of ESBL *E. coli* and *Klebsiella* species isolated from bovine mastitis cases are rare.

Ceftiofur resistance proportion (0.8%) and MIC_{90} value (0.5 µg/mL) in *E. coli* in our study was similar to those in the Finnish and Norwegian study (0%, 0.5 µg/mL) (FINRES-Vet, 2007; NORM/NORM-VET, 2009). However, ceftiofur resistance proportion in *E. coli*

(0.8%) and *Klebsiella* (0%) species was far lower than in a US study by Erskine et al. (2002) where 4.6% of *E. coli* and 14.1% of *Klebsiella* species isolates were resistant.

Fluoroquinolones are commonly used in companion animals. They have excellent activity against Enterobacteriaceae. Ciprofloxacin is used an indicator to test susceptibility to various fluoroquinolones. Ciprofloxacin is used extra-label in small animals whereas enrofloxacin and danofloxacin are approved for use in both small animals and cattle, respectively (Walker and Dowling, 2006). However, the extra-label use of fluoroquinolones in food animals is strongly discouraged in North America. Resistance to nalidixic acid was nearly absent in *E. coli* (0.2%) whereas all *E. coli* were sensitive to ciprofloxacin. Similar results were observed in most studies (Botrel et al., 2010; FINRES-Vet, 2007; Lanz et al., 2003; Lehtolainen et al., 2003; MARAN, 2008; NORM/NORM-VET, 2009; Srinivasan et al., 2007). However, MIC_{90} values of ciprofloxacin ($\leq$ 0.5 µg/mL) and nalidixic acid (2.0 µg/mL) were relatively lower in the present study. In general, resistance to nalidixic acid and ciprofloxacin is nearly absent in *E. coli*. All *Klebsiella* species isolates were also susceptible to nalidixic acid and ciprofloxacin. In general, resistance to antimicrobials of very high importance to human medicine (as categorized by Health Canada, (2009)) such as third-generation cephalosporins (ceftiofur, ceftriaxone), penicillin-ß-lactamase inhibitor (amoxicillin-CLA combination) and fluoroquinolones (ciprofloxacin) is uncommon to rare in bovine mastitis coliforms.

Multi-drug resistance was frequently found in resistant *E. coli* isolates. The vast majority of MDR *E. coli* were resistant to kanamycin, streptomycin, ampicillin, sulfisoxazole, TMP-sulfamethoxazole combination and tetracycline. Linkage between resistance determinants

of sulfonamides (*sul1* and *sul2* genes) and TMP (*dfr1 gene*) or streptomycin (*aadA1*) is very common; co-location of resistance determinants on specific plasmids thereby leading to linkage of resistance genes is a likely potential reason for such a large number of MDR patterns in *E. coli* isolates (Lanz et al., 2003). Multi-drug resistance to ampicillin, streptomycin, sulfisoxazole and tetracycline was also very commonly seen in *E. coli* isolated from bovine mastitis cases in the study by Srinivasan et al. (2007). However, proportion of MDR isolates was far higher in their study (90.7%). Lehtolainen et al. (2003) had also reported tetracycline-dihydrostreptomycin-ampicillin as the most frequent AMR pattern in MDR *E. coli* isolated from acute clinical bovine mastitis cases in Israel and Finland in their study, thereby potentially indicating transferable resistance mechanisms (Oppegard et al., 2001). In general, the occurrence of MDR *E. coli* isolated from bovine mastitis isolates needs to be monitored in the coming years as advised by Dutch and Norwegian AMR surveillance programs (MARAN, 2008; NORM/NORM-VET, 2009).

In contrast, the majority of MDR *Klebsiella* species isolates were resistant to two antimicrobials. Streptomycin–tetracycline, streptomycin–sulfisoxazole, and cefoxitin–amoxicillin-CLA combination resistance pattern was most commonly seen in *Klebsiella* species. Resistance to 7 antimicrobials such as chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, TMP-sulfamethoxazole combination and tetracycline was only observed in 2 isolates. A single plasmid conferring resistance to multiple antimicrobial drug classes could be the potential reason (Dowling, 2006).

Generally speaking, prevalence of AMR in udder pathogens is relatively low. Similar observations have been made globally (Bengtsson et al., 2009; Erskine et al., 2002;

Makovec and Ruegg, 2003). Commonly employed “full-dose short-term” treatment regimens are unlikely to promote dissemination of resistance determinants in an udder environment (WHO, 1997) and could be a potential reason for lower prevalence of AMR in bovine mastitis pathogens.

4.6 Conclusions

In general, AMR prevalence was uncommon in the bovine mastitis pathogens *S. aureus*, *E. coli*, and *Klebsiella* species. Differences in resistance proportions between isolates from IMI, subclinical and clinical mastitis cases were not evident. Multi-drug resistance was more commonly seen in *E. coli* and *Klebsiella* species than in *S. aureus*. Resistance to antimicrobials of very high importance to human medicine was rare in bovine mastitis isolates. Only 1 out of 1802 *S. aureus* isolates screened for MRSA was positive (prevalence 0.05%), and no ESBL *E. coli* and *Klebsiella* species were found in Canadian milk samples. The study results suggest a low risk of transmission of antimicrobial resistant bacteria from milk or milk products to human populations in Canada.

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Table 4.1: Distribution of minimum inhibitory concentrations (MIC) of *Staphylococcus aureus* (n=562) isolated from udders of dairy cattle on 79 dairy farms across 6 provinces in Canada.

	MIC (µg/mL)														
Antimicrobial	≤ 0.12	0.25	0.50	1	2	4	8	16	32	64	128	256	> 256	MIC_{50}	MIC_{90}
Ampicillin	92.4	5.0	2.2	0.2			0.2							≤ 0.12	≤ 0.12
Penicillin	91.2	6.8	1.0	1.0										≤ 0.12	≤ 0.12
Erythromycin		94.1	5.2				**0.7**							≤ 0.25	≤ 0.25
Oxacillin					100									≤ 2	≤ 2
Pirlimycin			94.8	2.3	0.5	1.2	**1.2**							≤ 0.50	≤ 0.50
Penicillin-novobiocin				98.9	0.5		0.2	**0.4**						≤ 1	≤ 1
Tetracycline				97.0		0.2	0.2	**2.6**						≤ 1	≤ 1
Cephalothin					99.8	0.2								≤ 2	≤ 2
Ceftiofur			45.1	50.3	4.3		**0.3**							1	1
Sulfadimethoxine									56.1	18.6	10.9	7.4	**7.0**	≤ 32	256

Note: Numbers indicate percentage of isolates. White areas indicate range of dilutions tested for each antimicrobial agent. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range. Vertical lines indicate clinical breakpoints with values to the left of the line being sensitive or intermediate and those to the right being resistant. MIC_{50} and MIC_{90} values are concentrations at which ≥ 50% and ≥ 90% of isolates are inhibited.

Table 4.2: Minimum inhibitory concentrations (MIC, µg/mL) and resistance proportions (R) of *Staphylococcus aureus* (n=562) isolated from intramammary infections (IMI), subclinical and clinical mastitis cases in dairy cattle on 79 dairy farms across 6 provinces in Canada.

	Overall (n=562)		IMI (n=118)			Subclinical mastitis (n=250)			Clinical mastitis (n=183)			
Antimicrobial	MIC Range	R (%) (95% CI)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	*P*-value[1]
Ampicillin	≤ 0.12 to 1	2.6 (1.4 to 4.3)	≤ 0.12	≤ 0.12	2.5	≤ 0.12	≤ 0.12	3.6	≤ 0.12	≤ 0.12	1.1	0.23
Penicillin	≤ 0.12 to 1	8.8 (6.6 to 11.5)	≤ 0.12	≤ 0.12	11.2	≤ 0.12	≤ 0.12	8.9	≤ 0.12	≤ 0.12	6.7	0.40
Erythromycin	≤ 0.25 to > 4	0.7 (0.1 to 1.7)	≤ 0.25	≤ 0.25	0	≤ 0.25	≤ 0.25	0.4	≤ 0.25	≤ 0.25	1.6	0.27
Oxacillin	- -	0	≤ 2	≤ 2	0	≤ 2	≤ 2	0	≤ 2	≤ 2	0	- -
Pirlimycin	≤ 0.50 to > 4	2.4 (1.3 to 4.1)	≤ 0.50	≤ 0.50	1.7	≤ 0.50	≤ 0.50	2.8	≤ 0.50	≤ 0.50	2.8	0.88
Penicillin-novobiocin	≤ 1 to > 8	0.6 (0.1 to 1.5)	≤ 1	≤ 1	0	≤ 1	≤ 1	0.4	≤ 1	≤ 1	1.1	0.59
Tetracycline	≤ 1 to > 8	2.6 (1.4 to 4.3)	≤ 1	≤ 1	1.7	≤ 1	≤ 1	2.0	≤ 1	≤ 1	3.9	0.49
Cephalothin	≤ 2 to 4	0	≤ 2	≤ 2	0	≤ 2	≤ 2	0	≤ 2	≤ 2	0	- -
Ceftiofur	≤ 0.50 to > 4	0.3 (0.04 to 1.2)	1	1	0	1	1	0	1	1	1.1	0.15
Sulfadimethoxine	≤ 32 to > 256	7.0 (5.0 to 9.4)	≤ 32	256	5.9	≤ 32	256	10.0	≤ 32	256	4.4	0.07

[1]*P*-value of an association between AMR profiles (Sensitive, Resistant) and source of isolates (IMI, subclinical mastitis, clinical mastitis cases).

Table 4.3: Distribution of minimum inhibitory concentrations (MIC) of *Escherichia coli* (n=394) isolated from udders of dairy cattle on 76 dairy farms across 6 provinces in Canada.

	MIC (µg/mL)																	
Antimicrobial	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC_{50}	MIC_{90}
Amikacin						0.5	15	67.6	14.7	1.6	0.3			**0.3**			2	4
Ampicillin							15.5	47.3	27.0	0.5	1.5	0.7	**7.5**				2	8
Amoxi-CLA[1]							5.0	34.4	45.9	11.5	1.5	1.5	**0.2**				4	8
Cefoxitin							1.2	30.9	58.2	5.2	2.0	1.5	**1.0**				4	4
Ceftriaxone					98.0	0.2	0.5		0.5		0.8						≤ 0.25	≤ 0.25
Ceftiofur				4.0	37.1	55.3	1.8	0.5	0.5	0.5	**0.3**						0.5	0.5
Chloramphenicol								3.7	41.2	50.9	1.2		**3.0**				8	8
Gentamicin					8.3	62.0	27.6	1.7		0.2		**0.2**					0.5	1
Kanamycin										94.3	1.0		0.5	**4.2**			≤ 8	≤ 8
Streptomycin												91.2	3.5	**5.2**			≤ 32	≤ 32
Ciprofloxacin	99.0	0.6	0.2				0.2										≤	≤
Nalidixic acid						0.2	10.5	75.1	14.0				**0.2**				2	2
Sulfisoxazole											76.7	13.3	0.5	0.3		**9.2**	≤ 16	64
TMP-sulfa[2]				82.3	11.3	0.2	0.2	0.2		**5.8**							≤ 0.12	0.25
Tetracycline									84.2	1.0		5.8	**9.0**				≤ 4	32

[1]Amoxicillin–clavulanic acid combination.

[2]Trimethoprim–sulfamethoxazole combination.

Note: Numbers indicate percentage of isolates. White areas indicate range of dilutions tested for each antimicrobial agent. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range.

Vertical lines indicate clinical breakpoints with values to the left of the line being sensitive or intermediate and those to the right being resistant. MIC_{50} and MIC_{90} values are concentrations at which $\geq$ 50% and $\geq$ 90% of isolates are inhibited.

Table 4.4: Minimum inhibitory concentrations (MIC, µg/mL) and resistance proportions (R) of *Escherichia coli* isolated from intramammary infections (IMI), subclinical and clinical mastitis cases in dairy cattle on 76 dairy farms across 6 provinces in Canada.

Antimicrobial	Overall (n=394)		IMI (n=13)			Subclinical mastitis (n=38)			Clinical mastitis (n=326)			
	MIC Range	R (%) (95% CI)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	*P*-value[3]
Amikacin	≤ 0.5 to > 64	0.3 (0.01 to 1.3)	2	4	0	2	4	2.6	2	4	0	0.13
Ampicillin	≤ 1 to > 32	8.2 (5.7 to 11.4)	2	4	0	2	16	5.2	2	8	8.2	0.72
Amoxi-CLA[1]	≤ 1 to > 32	1.7 (0.7 to 3.5)	4	8	0	4	8	0	4	8	1.8	1.00
Cefoxitin	1 to > 32	2.5 (1.2 to 4.5)	4	4	0	4	8	2.6	4	4	2.1	0.69
Ceftriaxone	≤ 0.25 to 16	0	≤ 0.25	≤ 0.25	0	≤ 0.25	≤ 0.25	0	≤ 0.25	≤ 0.25	0	- -
Ceftiofur	≤ 0.12 to > 8	0.8 (0.15 to 2.1)	0.5	0.5	0	0.5	0.5	0	0.5	0.5	0.6	1.00
Chloramphenicol	≤ 2 to > 32	3.0 (1.5 to 5.1)	4	8	0	4	8	2.6	8	8	2.4	1.00
Gentamicin	≤ 0.25 to > 16	0.2 (0.01 to 1.3)	0.5	1	0	0.5	1	0	0.5	1	0.3	1.00
Kanamycin	≤ 8 to > 64	4.7 (2.8 to 7.3)	≤ 8	≤ 8	0	≤ 8	≤ 8	7.8	≤ 8	≤ 8	4.2	0.46
Streptomycin	≤ 32 to > 64	8.7 (6.1 to 11.9)	≤ 32	≤ 32	0	≤ 32	≤ 32	7.8	≤ 32	≤ 32	8.9	0.74
Ciprofloxacin	≤ 0.015 to 1	0	≤ 0.015	≤ 0.015	0	≤ 0.015	≤ 0.015	0	≤ 0.015	≤ 0.015	0	- -
Nalidixic Acid	≤ 0.5 to > 32	0.2 (0.01 to 1.3)	2	2	0	2	4	2.6	2	4	0	0.13
Sulfisoxazole	≤ 16 to > 256	9.2 (6.6 to 12.5)	≤ 16	32	0	≤ 16	> 256	10.5	≤ 16	32	8.9	0.68
TMP-sulfa[2]	≤ 0.12 to > 4	5.8 (3.6 to 8.5)	≤ 0.12	0.25	0	≤ 0.12	0.25	7.8	≤ 0.12	0.25	5.2	0.52
Tetracycline	≤ 4 to > 32	14.8 (11.4 to 18.9)	≤ 4	≤ 4	0	≤ 4	32	11.1	≤ 4	32	14.9	0.29

[1]Amoxicillin–clavulanic acid combination.

[2]Trimethoprim–sulfamethoxazole combination.

[3]*P*-value of an association between AMR profiles (Sensitive, Resistant) and source of isolates (IMI, subclinical mastitis, clinical mastitis cases).

Table 4.5: Distribution of minimum inhibitory concentrations (MIC) of *Klebsiella* species (n=139) isolated from udders of dairy cattle on 37 dairy farms across 6 provinces in Canada.

	MIC (µg/mL)																	
Antimicrobial	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC_{50}	MIC_{90}
Amikacin						1.4	85.5	12.4	0.7								1	2
Ampicillin												82.7	**17.3**				32	> 32
Amoxi-CLA[1]							13.1	66.9	9.7	3.4	1.4	2.1	**3.4**				2	8
Cefoxitin							6.2	65.5	19.3	1.4		1.4	**6.2**				2	4
Ceftriaxone					98.6	0.7				0.7							≤ 0.25	≤ 0.25
Ceftiofur				0.7	10.3	79.3	8.3	0.7	0.7								0.5	0.5
Chloramphenicol								15.1	49.0	33.1	1.4		**1.4**				4	8
Gentamicin					78.7	17.2	2.7					**1.4**					≤ 0.25	0.5
Kanamycin										93.9		2.0	2.8	**1.3**			≤ 8	≤ 8
Streptomycin												85.5	11.0	**3.5**			≤ 32	64
Ciprofloxacin	63.4		35.2	0.7	0.7												≤ 0.015	0.03
Nalidixic acid							4.1	86.2	9.7								2	2
Sulfisoxazole											60.0	20.7	4.8	2.8		**11.7**	≤ 16	> 256
TMP-sulfa[2]				67.6	29.7	0.7				**2.0**							≤ 0.12	0.25
Tetracycline									80.7	0.7		4.1	**14.5**				≤ 4	> 32

[1]Amoxicillin–clavulanic acid combination.

[2]Trimethoprim–sulfamethoxazole combination.

Note: Numbers indicate percentage of isolates. White areas indicate range of dilutions tested for each antimicrobial agent. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range.

Vertical lines indicate clinical breakpoints with values to the left of the line being sensitive or intermediate and those to the right being resistant. MIC_{50} and MIC_{90} values are concentrations at which $\geq$ 50% and $\geq$ 90% of isolates are inhibited.

Table 4.6: Minimum inhibitory concentrations (MIC, µg/mL) and resistance proportions (R) of *Klebsiella* species isolated from intramammary infections (IMI), subclinical and clinical mastitis cases in dairy cattle on 37 dairy farms across 6 provinces in Canada.

Antimicrobial	Overall (n=139)		IMI (n=9)			Subclinical mastitis (n=18)			Clinical mastitis (n=109)			
	MIC Range	R (%) (95% CI)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	*P*-value[3]
Amikacin	≤ 0.5 to 4	0	1	1	0	1	1	0	1	2	0	- -
Ampicillin	≤ 32 to > 32	100	- -	- -	100	32	32	100	32	> 32	100	- -
Amoxi-CLA[1]	≤ 1 to > 32	5.5 (2.4 to 10.5)	2	2	0	2	> 32	16.6	2	8	3.6	0.12
Cefoxitin	1 to > 32	7.6 (3.8 to 13.1)	2	4	0	2	> 32	16.6	2	4	6.4	0.25
Ceftriaxone	≤ 0.25 to 8	0	≤ 0.25	8	0	≤ 0.25	≤ 0.25	0	≤ 0.25	≤ 0.25	0	- -
Ceftiofur	≤ 0.12 to 4	0	0.5	4	0	0.5	1	0	0.5	0.5	0	- -
Chloramphenicol	≤ 2 to > 32	1.4 (0.2 to 4.8)	4	8	0	4	8	0	4	8	1.8	1.00
Gentamicin	≤ 0.25 to > 16	1.4 (0.2 to 4.8)	≤ 0.25	≤ 0.25	0	≤ 0.25	≤ 0.25	0	≤ 0.25	0.5	1.8	1.00
Kanamycin	≤ 8 to > 64	4.1 (1.5 to 8.7)	≤ 8	≤ 8	0	≤ 8	32	5.5	≤ 8	≤ 8	4.5	1.00
Streptomycin	≤ 32 to > 64	14.5 (9.1 to 21.2)	≤ 32	≤ 32	0	≤ 32	64	11.1	≤ 32	64	17.4	0.48
Ciprofloxacin	≤ 0.015 to 0.12	0	≤ 0.015	0.12	0	≤ 0.015	0.03	0	≤ 0.015	0.03	0	- -
Nalidixic acid	1 to 4	0	2	4	0	2	2	0	2	4	0	- -
Sulfisoxazole	≤ 16 to > 256	11.7 (6.9 to 18.1)	≤ 16	128	0	≤ 16	> 256	11.1	≤ 16	> 256	12.8	0.77
TMP-sulfa[2]	≤ 0.12 to > 4	2.0 (0.4 to 5.9)	≤ 0.12	≤ 0.12	0	≤ 0.12	0.25	0	≤ 0.12	0.25	2.7	1.00
Tetracycline	≤ 4 to > 32	18.6 (12.6 to 25.9)	8	32	44.4	≤ 4	> 32	16.6	≤ 4	> 32	18.3	0.11

[1]Amoxicillin–clavulanic acid combination.

[2]Trimethoprim–sulfamethoxazole combination.

[3]*P*-value of an association between AMR profiles (Sensitive, Resistant) and source of isolates (IMI, subclinical and clinical mastitis cases).

Table 4.7: Multidrug resistance patterns in *Escherichia coli* (n=394) and *Klebsiella* species (n=139) isolated from udders of dairy cows across 6 provinces in Canada.

	Escherichia coli		*Klebsiella* species [a]	
Multidrug resistance pattern	# isolates[1]	% isolates[2]	# isolates[3]	% isolates[4]
AMP–AMX–CHL–XNL–FOX–KAN–STR–FIS–SXT–TET	1 (0.2)	1.4	- -	- -
AMP–AMX–CHL–XNL–FOX–STR–FIS–TET	1 (0.2)	1.4	- -	- -
AMP–AMX–FOX–KAN–STR–FIS–TET	2 (0.5)	2.8	- -	- -
AMP–CHL–KAN–STR–FIS–SXT–TET	2 (0.5)	2.8	- -	- -
AMP–FOX–KAN–NAL–STR–FIS–TET	1 (0.2)	1.4	- -	- -
AMP–AMX–XNL–KAN–STR–FIS–TET	1 (0.2)	1.4	- -	- -
CHL–GEN–KAN–STR–FIS–SXT–TET	- -	- -	2 (1.4)	4.0
AMP–KAN–STR–FIS–SXT–TET	7 (1.7)	10.0	- -	- -
AMP–AMX–KAN–STR–FIS–TET	2 (0.5)	2.8	- -	- -
CHL–GEN–KAN–FIS–SXT–TET	1 (0.2)	1.4	- -	- -
CHL–KAN–STR–FIS–SXT–TET	1 (0.2)	1.4	- -	- -
AMP–CHL–STR–FIS–SXT–TET	1 (0.2)	1.4	- -	- -
AMP–STR–FIS–SXT–TET	4 (1.0)	5.7	- -	- -
AMP–CHL–FIS–SXT–TET	2 (0.5)	2.8	- -	- -
AMP–CHL–FIS–TET	3 (0.8)	4.2	- -	- -
AMP–KAN–STR–FIS	1 (0.2)	1.4	- -	- -
KAN–FIS–SXT–TET	- -	- -	1 (0.7)	1.9
AMX–FOX–FIS	- -	- -	1 (0.7)	1.9
STR–FIS–TET	3 (0.8)	4.2	2 (1.4)	4.0
FIS–SXT–TET	1 (0.2)	1.4	- -	- -
AMX–FIS	- -	- -	1 (0.7)	1.9

AMX–FOX	- -	- -	7 (5.0)	13.7
STR–TET	5 (1.3)	7.1	6 (4.3)	11.8
FOX–TET			1 (0.7)	1.9
FIS–TET	2 (0.5)	2.8	1 (0.7)	1.9
STR–FIS	- -	- -	6 (4.3)	11.8
FIS–SXT	1 (0.2)	1.4	- -	- -
AMP–STR	1 (0.2)	1.4	- -	- -
AMP–KAN	1 (0.2)	1.4	- -	- -

AMK = amikacin; AMP = ampicillin; AMX = amoxicillin–clavulanic acid; FOX = cefoxitin; XNL = ceftiofur; AXO = ceftriaxone; CHL = chloramphenicol; CIP = ciprofloxacin; GEN = gentamicin; KAN = kanamycin; NAL = nalidixic acid; STR = streptomycin; FIS = Sulfisoxazole; TET = tetracycline; SXT = trimethoprim–sulfamethoxazole combination.

[a] Ampicillin resistance in *Klebsiella* species isolates was not accounted for due to intrinsic resistance.

[1]Denominator is 394 = total number of isolates tested.

[2]Denominator is 70 = total number of resistant isolates.

[3]Denominator is 139 = total number of isolates tested.

[4]Denominator is 51 = total number of resistant isolates.

Chapter Five: Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis *Staphylococcus aureus* isolates on Canadian dairy farms

5.1 Abstract

Surveillance of antimicrobial use and resistance is needed to manage antimicrobial resistance in bacteria. The present study collected data on antimicrobial use and resistance in *Staphylococcus aureus* (n=562) isolated from intramammary infections and (sub) clinical bovine mastitis cases on 89 dairy farms in four regions of Canada. Dairy producers and farm personnel were asked to deposit empty drug containers into specially provided receptacles and antimicrobial drug use rate was calculated to quantify antimicrobial use. MIC were determined using Sensititre® bovine mastitis plate containing antimicrobials commonly used for mastitis treatment and control. Multivariable logistic regression models were built to determine herd-level risk factors of penicillin, ampicillin, pirlimycin, penicillin-novobiocin combination, tetracycline and sulfadimethoxine resistance in *S. aureus* isolates.

Prevalence of antimicrobial resistance in *S. aureus* isolates was 20.4% (95% CI: 17.2 to 24.0%; range: 0% for cephalothin and oxacillin – 8.8% for penicillin). Intramammary administration of penicillin-novobiocin combination for dry cow therapy was associated with penicillin and ampicillin resistance (Odds Ratio [OR]: 2.17 and 3.10, respectively). Systemic administration of penicillin was also associated with penicillin resistance (OR: 1.63). Intramammary administration of pirlimycin for lactating cow mastitis treatment was associated with pirlimycin resistance as well (OR: 2.07). Average herd parity was associated with ampicillin and tetracycline resistance (OR: 3.88 and 0.02, respectively). Average herd size was also associated with tetracycline resistance (OR: 1.02). Dairy herds

in the Maritime region had higher odds of penicillin and lower odds of ampicillin resistance than dairy herds in Québec (OR: 2.18 and 0.19, respectively). Alberta dairy herds had lower odds of ampicillin and sulfadimethoxine resistance than dairy herds in Québec (OR: 0.04 and 0.08, respectively). Ontario dairy herds had lower odds of tetracycline and sulfadimethoxine resistance than dairy herds in Québec (OR: 0.05 and 0.33, respectively).

In conclusion, herd-level use of certain antimicrobials administered for mastitis treatment and control such as intramammary penicillin and pirlimycin as well as systemically administered penicillin and florfenicol was positively associated with antimicrobial resistance in bovine mastitis pathogens in the field conditions. Differences in antimicrobial resistance outcomes across four regions of Canada were observed.

5.2 Introduction

Antimicrobial use in humans and animals is considered a primary cause of antimicrobial resistance (AMR) in bacteria, which is a public health hazard (Levy and Marshall, 2004). There are growing concerns about the use of antimicrobials in food-animal production systems and its potential role in creating reservoirs of AMR determinants that can be transferred from animal to human populations along the food chain (Tikofsky et al., 2003, White and McDermott, 2001). Dairy farms form an ideal environment in which bacteria are subjected to antimicrobial treatments, and the subsequent selection pressure might favor selection and dissemination of resistant strains (Acar and Moulin, 2006, Silbergeld et al., 2008). It is therefore important to identify and measure factors impacting antimicrobial use (AMU) and AMR in a dairy farm environment for both clinical and public health reasons.

Mastitis is the primary reason for antimicrobial use in dairy cattle (Mitchell et al., 1998). A variety of mastitis pathogens *Staphylococcus aureus*, *Streptococcus* species, *Escherichia coli*, *Klebsiella* species, and coagulase-negative staphylococi are commonly isolated from bovine intramammary infections (IMI) (Olde Riekerink et al., 2008; Piepers et al., 2007). *S. aureus* remains one of the most important causes of clinical mastitis, and the most frequently isolated pathogen in subclinical mastitis cases worldwide (Waage, 1997; Barkema et al., 1998; Roberson et al., 1998; Olde Riekerink et al., 2008; Sampimon et al., 2009). Antimicrobial therapy is the preferred approach for decreasing the incidence and duration of mastitis infection on a dairy farm (Erskine et al., 2002). Cephalosporins, penicillins, penicillin-combinations, lincosamides, and macrolides are most commonly

administered intramammarily either during the lactation or dry period for treatment and/or prevention of mastitis in dairy cattle (Saini et al., 2011b). However, despite best possible antimicrobial treatments, bacteriological cure failures are common in *S. aureus* mastitis and AMR is considered to be one of the reasons for low cure rates (Barkema et al., 2006). Resistance to various antimicrobials is commonly seen in bovine *S. aureus* mastitis isolates. For example, prevalence of AMR has ranged from 7 to 63% for penicillin (Watts and Salmon, 1997; Güler et al., 2010), 0 to 12% for oxacillin (Saini et al., 2011a; FINRES-Vet, 2007), 0 to 93% for erythromycin (FINRES-Vet, 2007; Wang et al., 2008), 0 to 28% for tetracycline (Watts and Salmon, 1997; Güler et al., 2010) and 4.5 to 7.5% for sulfadimethoxine (Makovec and Ruegg, 2003; Sabour et al., 2004) across various studies. In general, ß-lactamase negative *S. aureus* strains have higher bacteriological cure rates than ß-lactamase producing stains (Sol et al., 2000; Ziv and Storper, 1985).

A few studies have assessed an association between AMU and AMR in bovine mastitis pathogens with conflicting results (Tikofsky et al., 2003; Rajala-Schultz et al., 2004; Roesch et al., 2006; Pol and Ruegg, 2007). Tikofsky et al. (2003) compared antimicrobial susceptibility patterns of *S. aureus* in organic and conventional dairy herds in the US, and found that isolates from organic herds were significantly more susceptible. No such differences were found in a Swiss study while comparing antimicrobial susceptibility of *S. aureus* isolated from organic and conventional farms (Roesch et al., 2006). Pol and Ruegg (2007) collected information on herd-level AMU and antimicrobial susceptibility profiles of Gram-positive mastitis pathogens including *S. aureus* on organic and conventional dairy farms; the researchers found association between AMU for mastitis treatment and control and AMR for some antimicrobials. None of these studies modeled AMR in bovine mastitis

pathogens by including AMU (quantity, route of administration) and other herd-level predictors (region, milk production, barn type, SCC, parity, source of isolates [subclinical, clinical mastitis, IMI], severity of mastitis [mild, moderate, severe]) that could potentially impact AMU-AMR association. In fact, there is a lack of convincing evidence that AMU for mastitis treatment and control is associated with AMR in bovine mastitis pathogens in a dairy farm environment (Hillerton and Berry, 2005).

The objective of the present study was to determine the herd-level association between AMU and other herd-level predictors, and AMR in *S. aureus* isolated from IMI and (sub) clinical bovine mastitis cases on Canadian dairy farms.

5.3 Materials and Methods

5.3.1 Herd Selection

Data for this study originated from the National Cohort of Dairy Farms of the Canadian Bovine Mastitis Research Network (CBMRN), which consisted of 91 commercial dairy farms located in four regions across Canada (Alberta, Ontario Québec, and the Maritime Provinces [Prince Edward Island, Nova Scotia, and New Brunswick]). Herd selection criteria for the present study have been described by (Reyher et al., 2011). In short, dairy herds were selected to replicate the regional proportion of free-stall systems to within 15 percentage points and to be uniformly distributed among three strata of the most recent 12-month bulk tank SCC average (≤ 150,000 cells/mL, > 150,000 and ≤ 300,000 cells/mL, > 300,000 cells/mL); herds with three times milking schedule per day and herds with less than 80% (or less than 15) Holstein lactating and dry cows at the time of enrolment were excluded. Further, eligible dairy herds must have been participating in a DHI data

collection program. Eligible dairy farms were identified and contacted by the regional center coordinators. Written consent to participate in the research cohort was obtained. Two dairy herds dropped out of the study at the very beginning leaving 89 herds in the study. An investigator and technicians in each coordinating center were responsible for the data collection activities related to remaining farms located in that center's area.

5.3.2 Antimicrobial Use Data Collection Methodology

Antimicrobial use data collection has been described by Saini et al. (2011b). In short, AMU data were collected from February 2007 until December 2008. Forty-liter receptacles were placed on participating farms for collecting data for AMU. These receptacles were placed near the drug storage area, in the milking parlour or any place near where the treatments were normally given. Producers, farm workers and other farm personnel were instructed to deposit the empty containers of all drugs used by them or the veterinarian for treatment in calves, heifers and adult cows (dry and lactating) into these receptacles. Farms were visited at least once per month. The research technicians emptied the receptacles, counted the empty drug bottles and recorded the inventory in the drug tally sheets at the dairy farm.

Antimicrobial use data were quantified in units of animal defined-daily doses (ADD). The ADD (g/day) was defined as the average daily on-label dosage multiplied by the approximate weight of an adult dairy cow (BW=600 kg) (Jensen et al., 2004) and was based on the Canadian compendium of veterinary products. Further, antimicrobial drug use rate (ADUR) was defined as number of ADD used on a farm per 1,000 cows (milking and dry) per day. ADUR was the herd-level estimate of AMU (Table 5.1).

5.3.3 Sampling and Bacterial Culturing

Sampling and bacterial culturing of isolates has been described elsewhere (Reyher et al., 2011). In short, three different sets of milk samples were collected. The first set included milk samples from clinical mastitis cases. All producer-diagnosed clinical mastitis cases were sampled and then re-sampled twice at two-week interval. The second set was from milk samples from nonclinical lactating cows; a sub-sample of 15 fresh and lactating cows was selected per farm. They were aseptically sampled and re-sampled once every 3 weeks for a total of three samplings in the winter of 2007; another sub-sample of 15 lactating cows was sampled once a week for 7 weeks in the summer of 2007.

The third set of milk samples were collected from another selected group of 15 cows that were expected to remain in the herd until at least 2 weeks after calving. A sub-sample was aseptically sampled before dry-off and after calving in 2007 and this continued in 2008 as well. Quarter and composite milk samples were collected. Samples were frozen at -20^0C and shipped to the regional CBMRN laboratory where bacterial culturing and identification of the milk samples was done as per National Mastitis Council guidelines (Hogan et al., 1999).

Because multiple isolates could be coming from a single cow, it was decided to include only one isolate per quarter. Clinical mastitis was defined as an inflammation of the udder leading to occurrence of flakes, clots or other gross alterations in milk. Subclinical mastitis was defined as SCC >200,000 cells/mL from a cow without clinical signs of mastitis, whereas IMI was defined as a culture-positive sample (Reyher et al., 2011).

5.3.4 MIC Determination

Antimicrobial susceptibility testing of these *S. aureus* isolates has been described elsewhere (Saini et al., 2011a). Minimum inhibitory concentrations (MIC) of these isolates were determined using the Sensititre® microdilution system (TREK Diagnostic Systems Inc., Cleveland, Ohio). Sensititre® Mastitis plate format CMV1AMAF containing ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, penicillin-novobiocin combination, pirlimycin, sulfadimethoxine, and tetracycline was used.

5.3.5 Statistical Analyses

Antimicrobial use data were entered into a customized database (Microsoft Office Access 2006, Microsoft Corporation, Redmond, Washington, USA). A random sample of the drug tally sheets (25%) was checked manually to detect errors in data entry. Data analyses were performed using Intercooled Stata®11.1 (Intercooled Stata for Macintosh, version 11.1, Stata Corporation, College Station, TX).

Minimum inhibitory concentration was defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate. In case of an antimicrobial combination such as penicillin-novobiocin, the MIC of the first agent (penicillin) was reported as the MIC for the combination. The isolates were also categorized as sensitive, intermediate and resistant on the basis of CLSI based MIC breakpoints (2008). Intermediate isolates were combined with resistant category isolates for the sake of statistical analysis. For analytical purposes, the unit of analysis was resistance at the herd-level (0: number of resistant isolates at a farm = 0; and 1: number of resistant isolates at a farm $\geq$ 1). Herd-level

prevalence percentage of AMR was calculated and defined as percentage of total number of herds with AMR isolates divided by total number of herds sampled.

All independent variables (antimicrobial use and other herd-level factors such as region, herd average milk production, barn type, herd average SCC, herd average parity, average herd size, number of isolates sampled at a farm) were screened based on descriptive statistics (means, variances and percentiles for continuous variables, and frequency tabulations for categorical variables) so as to exclude variables that had little variability (Dohoo et al., 2009). Subsequently, these independent variables were screened for univariate associations using the Likelihood Ratio Test Statistic (Hosmer and Lemeshow, 2000). Variables significant at $P \leq 0.25$ were eligible for inclusion in the multivariable logistic regression models. Using backward elimination, variables were retained in models only if significant at $P \leq 0.05$ unless exclusion resulted in significant change in deviance. Thereafter, any variables not selected for the original multivariable model were added back into the model in order to identify variables that were not significantly associated with the herd-level outcome, but made an important contribution in the presence of other variables (distorter variables). Barn type, region, average herd size, and average herd parity were considered *a priori* as potential confounders. Variables as average herd SCC, herd average milk production per cow, average herd size and average herd parity were centered at their respective lowest values for sensible interpretation of the intercept value.

The assumption of linearity in the logit of herd-level AMR outcome for continuous variables was evaluated graphically using lowess smoother scatter plots (Dohoo et al., 2009) and then by using fractional polynomials command in Intercooled Stata®11.1. Two-

way interaction terms were added one at a time to the main effects model and retained at $P \leq 0.05$ unless exclusion resulted in significant change in deviance. Robust standard errors were used to control for clustering of farms within regions. In case of nominal variables such as region, the baseline/referent level was selected as the one with sufficiently large sample size (Dohoo et al., 2009).

Multivariable logistic regression models were built for the following herd-level AMR outcomes: penicillin, ampicillin, pirlimycin, penicillin-novobiocin combination, tetracycline and sulfadimethoxine. No models could be built for oxacillin and cephalothin due to absence of resistance, and for ceftiofur and erythromycin due to low prevalence of herd-level resistance.

5.4 Results

5.4.1 Antimicrobial Resistance Proportions

Minimum inhibitory concentration values were determined for 562 isolates that came from 562 quarters of 462 cows on 79 dairy farms. Total numbers of isolates resistant to one or more antimicrobials were 114 or a prevalence of 20.4% (95% CI: 17.2 to 24.0%). Total number of isolates resistant to an antimicrobial ranged from 0 to 9 per farm. Herd-level AMR prevalence proportions ranged from 0% for cephalothin and oxacillin to 35.4% for penicillin and sulfadimethoxine (Table 5.2).

5.4.2 Multivariable Analyses

The odds for herds having at least one penicillin-resistant *S. aureus* isolate increased with an increasing use of penicillin-novobiocin combination administered for dry cow therapy

and systemically administered penicillin (Table 5.3). Dairy herds in the Maritime region had significantly higher odds of having at least one penicillin-resistant *S. aureus* than dairy herds in Québec.

The odds of ampicillin resistance at the herd-level increased significantly with increased use of penicillin-novobiocin combination administered for dry cow therapy and systemically administered florfenicol (Table 5.3). The odds of ampicillin resistance also increased significantly with an increase in average herd parity. Dairy herds in Alberta and Maritime region had significantly lower odds of ampicillin resistance than dairy herds in Québec.

The odds of pirlimycin resistance at the herd-level also increased significantly with increase in the use of pirlimycin administered for lactating cow mastitis treatment (Table 5.4). Odds of tetracycline resistance significantly decreased with increase in herd average parity. Odds of tetracycline resistance increased significantly with increase in average herd size. Dairy herds in Ontario had significantly lower odds of tetracycline resistance than dairy herds in Québec. Dairy herds in Ontario and Alberta had significantly lower odds of sulfadimethoxine resistance than dairy herds in Québec. No statistically significant interaction terms between explanatory variables were found.

5.5 Discussion

The present study has assessed and evaluated the impact of herd-level AMU on herds' AMR profile patterns in bovine mastitis *S. aureus* isolates. The study accounted for potential differences in antimicrobial use and susceptibility due to geographical and

epidemiological differences. This is the first time that a Canada-wide prospective study to quantify on-farm AMU, AMR profile patterns and to determine an association between the two for bovine mastitis *S. aureus* pathogens has been undertaken.

Antimicrobial susceptibility testing was done using Sensititre® MIC system. Sensititre® MIC and Kirby-Bauer disk diffusion test method have recently been validated for common bovine mastitis pathogens and were found to have a moderate to high diagnostic accuracy, and very good essential and categorical agreement for most udder pathogen-antimicrobial combinations (Saini et al., 2011c). In general, prevalence of AMR was low in our Canadian bovine mastitis *S. aureus* isolates. Selection, emergence and propagation of resistant bacterial population requires a) repeated exposure to an antimicrobial, b) access of bacterium to a large resistance gene pool in a multi-bacterial environment, and c) presence of mobile genetic elements (Schwarz and Chaslus-Dancla, 2001). The environment inside the udder is virtually sterile as compared to gastro-intestinal tract where no indigenous bacterial flora is present (Werckenthin et al., 2001), and most IMI usually contain a single pathogen thus limiting its exposure to resistant gene pools. Further, commonly employed "full-dose short-term" treatment regimens are unlikely to promote dissemination of resistance determinants in an udder environment (WHO, 1997) and could be a potential reason for lower prevalence of AMR in bovine mastitis pathogens.

Cross-resistance, potential co-selection and direct effects of AMU on AMR were observed in the present study. Direct effect means that AMU selects for AMR to the same antimicrobial e.g. systemically administered penicillin selected for penicillin resistance in *S. aureus*. Cross-resistance is observed when AMU selects for resistance to all

antimicrobials in the same class (e.g. sulfonamides), some antimicrobials in a class (e.g. aminoglycosides) or antimicrobials belonging to different classes (e.g. resistance to macrolides, lincosamides and B streptogramins) due to a single biochemical mechanism such as ß-lactamase production, target overlapping or unspecified drug efflux (Guardabassi and Courvalin, 2006). In this study, systemically administered penicillin cross-selected for ampicillin resistance. Potential co-selection is observed when AMU selects for resistance to an antimicrobial of a different class due to coexistence of genes or mutations in the same strain. Systemically administered florfenicol potentially co-selected for ampicillin resistance in the present study. These results indicate that use of some antimicrobials is positively associated with AMR in bovine mastitis pathogens in the field conditions.

Route of administration of an antimicrobial could potentially impact AMR in udder pathogens. For an antimicrobial to be effective, it must reach the site of infection in effective concentration. The bovine udder is rich in blood supply. However, the rate of passage of an antimicrobial into milk after parentral administration depends upon the degree of lipid solubility, and extent of plasma-protein binding (Baggot, 2006). In general, only the lipid-soluble, non-ionized and plasma-protein unbound fraction of an antimicrobial can penetrate blood-milk barrier to enter into milk and diffuse into transcellullar fluid. Penicillins are predominantly ionized in plasma, are less lipid-soluble and cross biological membranes poorly; the concentration in milk is about one-fifth of that in the serum (Prescott, 2006). Florfenicol is not labeled for use in lactating dairy cattle. However, it can be potentially useful in treating mastitis in lactating dairy cattle due to appropriate pharmacokinetics (Soback et al., 1995). In case of lactating dairy cattle, systemically administered florfenicol readily crosses into milk, however it has lower bioavailability than

when administered by intramammary route. It is therefore quite likely that sub-therapeutic concentrations of penicillin and florfenicol are achieved in the udder upon systemic administration that may cause selection of penicillin and ampicillin resistant *S. aureus* strains.

It has long been speculated that administration of antimicrobials in dry cow therapy is associated with emergence of resistance in bovine mastitis pathogens (Osterås et al., 1999). Present study results provide support for that hypothesis. Pol and Ruegg (2007) also found a positive association between AMU and AMR in *S. aureus* isolates in case of penicillin and ampicillin, and a positive correlation between pirlimycin use and MIC in their study (Pol and Ruegg, 2007). In the present study, intramammary administration of penicillin-novobiocin combination for dry cow treatment was associated positively with penicillin and ampicillin resistance. However, no associations were observed for cloxacillin, cephapirin, ceftiofur, and pirlimycin administered intramammary for dry cow and lactating cow therapy. Interestingly, systemic administration of florfenicol was positively associated with ampicillin resistance. This is surprising because florfenicol is not known to induce ß-lactamase production in *S. aureus*. Therefore, either this is a spurious association or indicative of resistance mechanisms such as co-selection that are yet to be identified in *S. aureus*.

In general, AMR tended to be associated with average herd parity and barn type. For example, odds of penicillin and ampicillin resistance at a farm increased with an increase in average herd parity. Similar observations were made for CNS isolated from milk samples obtained from primiparous and multiparous cows (Rajala-Schultz et al., 2004; Sol et al.,

2000). Increase in parity indicates an increase in age and therefore increased exposure to antimicrobials and mastitis pathogens. Age or body weight could also influence relative systemic availability of an antimicrobial, and that could potentially impact AMR as well. Similar observations have been made in calves (Marshall and Palmer, 1980). Further, except for tetracycline, prevalence of AMR in *S. aureus* tended to be higher in tie-stall barns than free-stall barns. It is quite likely that barn type is a surrogate for management practices that potentially impact AMU-AMR association in bovine mastitis pathogens. Incidence of IMI and/or (sub) clinical mastitis is the primary reason for AMU on a dairy farm. The authors advise that information about managemental factors impacting incidence of IMI and/or (sub) clinical mastitis on a dairy farm such as nutrition, milk production, leaking of milk, breed of cows, post-milking teat disinfection, housing, hygienic condition of cubicles and cows, and milking procedures (Barkema et al., 1999) should also be collected in studies determining risk factors of AMR.

The unit of analysis and concern in this AMU-AMR association study was the farm. Antimicrobial resistance is potentially a herd-level phenomenon as farm ecosystems provide an ideal environment for emergence, amplification, and dissemination of resistant bacteria/determinants (Acar and Moulin, 2006). Secondly, data were collected on herd-level use of antimicrobials; individual cow-level AMU data being unavailable, it will be a fallacy to make inferences from this study at individual cow-level. Dairy herds in this study were not randomly selected. However, these herds were representative of their respective dairy herd populations in Canada (Reyher et al., 2011). The results of this study can therefore be generalized to dairy herd populations in Canada.

5.6 Conclusions

Herd-level use of penicillin-novobiocin combination administered intramammary for dry cow therapy, and systemically administered penicillin was positively associated with penicillin resistance in *S. aureus* isolated from IMI and (sub) clinical bovine mastitis cases on Canadian dairy farms. Ampicillin resistance was associated with the use of systemically administered florfenicol. Intramammary administration of pirlimycin for clinical mastitis treatment was also associated with pirlimycin resistance. Average herd parity was associated with AMR to ampicillin and tetracycline. There were differences in AMR to certain antimicrobials across four regions that could not be explained by antimicrobial use data solely.

5.7 References

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Table 5.1: Descriptive statistics of herd-level factors and antimicrobial drug use rate (Animal defined-daily doses (ADD)/1000 cow-days) of selected antimicrobial drug classes used across 89 Canadian dairy farms in 6 Canadian provinces.

Explanatory variable	Description	Herds (%)	Mean	SEM	Min.	Median	Max.
Herd size	Average herd size	89 (100)	84	4.96	33	66	297
Somatic cell count	Geometric herd SCC mean (x 1000 cells/mL)	89 (100)	230	9.26	91	220	500
Milk production	Herd average milk production / cow (kg)	89 (100)	32	0.31	25	32	39
Parity	Average herd parity	89 (100)	2.5	0.03	1.5	2.5	3.2
			Antimicrobial drug use rate				
Cloxacillin	Intramammary use (dry cow therapy)	51 (57)	0.44	0.06	0	0.09	2.00
Cephapirin	Intramammary use (dry cow therapy)	42 (47)	0.29	0.05	0	0	1.92
Penicillin-novobiocin	Intramammary use (dry cow therapy)	54 (61)	0.80	0.09	0	0.45	2.76
Cephapirin	Intramammary use (lactating cow therapy)	64 (72)	0.64	0.11	0	0.29	6.66
Ceftiofur	Intramammary use (lactating cow therapy)	28 (31)	0.11	0.02	0	0	1.19
Penicillin Combination[1]	Intramammary use (lactating cow therapy)	84 (94)	2.31	0.30	0	1.73	19.68
Pirlimycin	Intramammary use (lactating cow therapy)	52 (58)	0.68	0.15	0	0.04	8.91
Ceftiofur	Systemic use	77 (86)	1.80	0.19	0	1.19	7.09
Penicillin	Systemic use	75 (84)	1.30	0.12	0	1.06	5.24
Tetracycline	Systemic use	56 (63)	0.69	0.10	0	0.31	4.13

TMP-sulfadoxine Combination	Systemic use	68 (76)	0.88	0.10	0	0.52	3.96
Florfenicol	Systemic use	39 (44)	0.14	0.03	0	0	1.21

[1]Contains Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

Table 5.2: Descriptive statistics of herd-level antimicrobial resistance outcomes in *Staphylococcus aureus* (n=562) isolated from intramammary infections (IMI), subclinical and clinical mastitis cases in dairy cattle on 79 dairy farms across 6 provinces in Canada.

Resistance Outcome	Herds[1] (%)	Isolates[2]	Mean[3]	SEM	Min.	Median	Max.
Penicillin	28 (35.4)	49	0.62	0.15	0	0	9
Ampicillin	12 (15.1)	15	0.18	0.05	0	0	2
Erythromycin	4 (5.0)	4	0.05	0.02	0	0	1
Oxacillin	0	0	--	--	--	--	--
Pirlimycin	6 (7.5)	14	0.17	0.11	0	0	9
Penicillin-novobiocin	6 (7.5)	6	0.07	0.02	0	0	1
Tetracycline	7 (8.8)	13	0.16	0.06	0	0	4
Cephalothin	0	0	--	--	--	--	--
Ceftiofur	2 (2.5)	2	0.02	0.01	0	0	1
Sulfadimethoxine	28 (35.4)	38	0.48	0.08	0	0	3

[1]Number of herds with antimicrobial resistant *Staphylococcus aureus* isolates.

[2]Number of antimicrobial resistant *Staphylococcus aureus* isolates.

[3]Average number of antimicrobial resistant *Staphylococcus aureus* isolates per farm.

Table 5.3: Final logistic regression models depicting herd-level association between antimicrobial use and antimicrobial resistance to penicillin and ampicillin in *Staphylococcus aureus* (n=562) isolated from bovine mastitis cases on 79 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Penicillin	Penicillin-novobiocin use (dry cow therapy)	2.17	0.56	0.003	1.30 to 3.61
	Penicillin use (systemic)	1.63	0.26	0.003	1.18 to 2.25
	Average herd size	1.003	0.01	0.79	0.98 to 1.02
	Average herd parity	1.76	1.85	0.58	0.22 to 13.80
	Barn type (0 - free stall, 1 - tie stall)	2.29	2.00	0.34	0.41 to 12.72
	Region:				
	Québec (baseline)	1.00			
	Ontario	1.25	0.41	0.50	0.65 to 2.40
	Alberta	0.29	0.19	0.058	0.08 to 1.04
	Maritimes	2.18	0.39	< 0.01	1.53 to 3.09
Ampicillin	Penicillin-novobiocin use (dry cow therapy)	3.10	0.60	< 0.01	2.21 to 4.55
	Penicillin use (systemic)	1.96	0.74	0.07	0.93 to 4.13
	Florfenicol use (systemic)	20.86	29.07	0.02	1.36 to 320.19
	Average herd size	1.003	0.01	0.85	0.96 to 1.04
	Average herd parity	3.88	2.19	< 0.01	1.28 to 11.76
	Barn type (0 - free stall, 1 - tie stall)	10.04	22.15	0.29	0.13 to 757.46
	Region:				
	Québec (baseline)	1.00			
	Ontario	0.66	0.20	0.19	0.36 to 1.22
	Alberta	0.04	0.06	0.04	0.002 to 0.86
	Maritimes	0.19	0.08	< 0.01	0.08 to 0.46

Table 5.4: Final logistic regression models depicting herd-level association between antimicrobial use and antimicrobial resistance to pirlimycin, penicillin-novobiocin combination and tetracycline in *Staphylococcus aureus* (n=562) isolated from bovine mastitis cases on 79 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Pirlimycin	Pirlimycin use (lactating cow therapy)	2.07	0.42	< 0.01	1.38 to 3.09
	Average herd size	1.00	0.01	0.65	0.97 to 1.04
	Average herd parity	0.34	0.57	0.52	0.01 to 9.03
	Barn type (0-free stall, 1- tie stall)	4.15	9.72	0.54	0.04 to 407.87
	Region:				
	Québec (baseline)	1.00			
	Ontario	0.91	0.43	0.85	0.36 to 2.31
	Alberta	Omitted due to absence of resistance			
	Maritimes	1.79	2.27	0.64	0.14 to 21.69
Penicillin-novobiocin Combination	Penicillin-novobiocin use (dry cow therapy)	1.23	0.56	0.65	0.49 to 3.04
	Florfenicol use (systemic)	34.56	92.95	0.18	0.17 to 6726
	Average herd size	1.00	0.01	0.74	0.96 to 1.04
	Average herd parity	12.63	28.56	0.26	0.15 to 1060
	Barn type (0-free stall, 1- tie stall)	1.25	2.48	0.90	0.02 to 60.52
	Region:				
	Québec (baseline)	1.00			
	Ontario	14.14	20.59	0.06	0.81 to 245.42
	Alberta	Omitted due to absence of resistance			
	Maritimes	6.85	9.09	0.14	0.50 to 92.21

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Tetracycline	Tetracycline use (systemic)	1.13	1.11	0.89	0.16 to 7.73
	Average herd size	1.02	0.008	0.005	1.007 to 1.03
	Average herd parity	0.02	0.02	< 0.01	0.004 to 0.13
	Barn type (0-free stall, 1- tie stall)	0.08	0.19	0.28	0.008 to 7.92
	Region:				
	Québec (baseline)	1.00			
	Ontario	0.05	0.029	< 0.01	0.018 to 0.15
	Alberta	Omitted due to absence of resistance			
	Maritimes	0.37	0.41	0.37	0.04 to 3.27
Sulfadimethoxine	TMPS[1] use (systemic)	0.83	0.29	0.60	0.41 to 1.66
	Average herd size	0.99	0.006	0.99	0.98 to 1.01
	Average herd parity	1.27	1.48	0.83	0.12 to 12.55
	Barn type (0-free stall, 1- tie stall)	1.05	1.35	0.96	0.08 to 13.02
	Region:				
	Québec (baseline)	1.00			
	Ontario	0.33	0.07	< 0.01	0.22 to 0.51
	Alberta	0.08	0.06	< 0.01	0.01 to 0.40
	Maritimes	0.40	0.24	0.13	0.12 to 1.34

[1]Trimethoprim-sulfadoxine combination.

Chapter Six: Relationship between antimicrobial use and antimicrobial resistance in Gram-negative bovine mastitis pathogens on Canadian dairy farms.

6.1 Abstract

Concurrent data on antimicrobial use and resistance are important for containing antimicrobial resistance in bacteria. The present study determined a herd-level association between antimicrobial use and resistance in *Escherichia coli* (n=394) and *Klebsiella* species (n=139) isolated from intramammary infections and bovine mastitis cases on 89 dairy farms in four regions of Canada. Antimicrobial use data were collected using inventory of empty antimicrobial containers, and antimicrobial drug use rate was calculated to quantify herd-level antimicrobial use. Minimum inhibitory concentrations were determined using Sensititre® NARMS Gram-negative plate. Multivariable logistic regression models were built to determine herd-level odds of resistance to tetracycline, ampicillin, ceftiofur, chloramphenicol, trimethoprim-sulfamethoxazole (TMPS) combination and streptomycin in coliforms.

Herd-level use of systemically administered tetracycline was associated with tetracycline resistance in *Klebsiella* species isolates (Odds ratio [OR] = 2.59). The use of systemically administered tetracycline was associated with tetracycline resistance in *E. coli* isolates on dairy farms using tetracycline and penicillin (OR = 3.61). Ampicillin resistance in *E. coli* isolates was associated with the use of systemically administered ceftiofur (OR = 21.33) and penicillin (OR = 3.73). Use of systemically administered TMPS was also associated with TMPS resistance in *E. coli* (OR = 1.68). The use of intramammary penicillin combination containing dihydrostreptomycin administered for clinical mastitis treatment and penicillin-novobiocin combination administered for dry cow therapy was associated

with streptomycin resistance as well (OR: 2.26, 1.75, respectively). Herds with tie-stall barns had higher odds of ceftiofur and ampicillin resistance in *E. coli* isolates than herds with free-stall barns (OR: 10.06, 14.15, respectively). Alberta dairy herds had lower odds of ceftiofur-resistant *E. coli* isolates than dairy herds in Ontario (OR: 0.13); however, dairy herds in Alberta had higher odds of TMPS, tetracycline, ampicillin, and chloramphenicol resistance in *E. coli* isolates than dairy herds in Québec (OR: 1.89, 2.54, 5.48, 20.27, respectively). Ontario dairy herds had lower odds of streptomycin resistance in *E. coli* isolates than dairy herds in Québec (OR: 0.64).

In conclusion, association between herd-level antimicrobial use and resistance in bovine mastitis coliforms was observed for certain antimicrobials. Barn type was positively associated with ceftiofur and ampicillin resistance in *E. coli*. Geographical variation in antimicrobial resistance was observed.

6.2 Introduction

Environmental mastitis in dairy cattle caused by coliforms occurs mostly in early lactation with high producing cows overrepresented. Coliform mastitis can cause more often severe systemic signs and occasionally death as compared to Gram-positive mastitis (Barkema et al., 1998). The majority of coliform mastitis cases are caused by *Escherichia coli* (Kaipainen et al., 2002; Lehtolainen et al., 2003). *Klebsiella* is another coliform organism that is an emerging pathogen with rising incidence in North America (Zadoks and Munoz, 2007); *Klebsiella* was the most frequently found clinical mastitis pathogen in free-stall dairy cattle herds in Western Canada (Olde Riekerink et al., 2008). These Gram-negative udder pathogens have been implicated in as low as 20% to more than 60% of clinical mastitis in different countries (Pyörälä and Honkanen-Buzalski, 1994; Shpigel et al., 1998). Association between incidence of coliform clinical mastitis and bulk tank SCC has also been observed. Incidence of coliform clinical mastitis and associated systemic signs was higher in herds with low bulk tank SCC as compared to herds with high bulk tank SCC (Barkema et al., 1998).

Broad-spectrum antimicrobials are commonly used to treat coliform mastitis (Erskine et al., 2002b; Kaipainen et al., 2002), although there is no convincing evidence of the efficacy of treatment (Sandholm et al., 1990; Erskine et al., 1992; Pyörälä et al., 1994); antimicrobial resistance (AMR) could be one potential reason for the lack of efficacy. Antimicrobials with an appropriate spectrum of activity against coliform bacteria that can be administered systemically in lactating dairy cattle include oxytetracycline, sulfadimethoxine, ceftiofur,

ampicillin, and amoxicillin (Wagner and Erskine, 2006). However, AMR in bovine mastitis coliforms is commonly observed. For example, resistance proportions in *E. coli* isolates ranged from 5 to 37% for tetracycline (FINRES-Vet, 2007; Makovec and Ruegg, 2003), 7 to 34% for sulfisoxazole (FINRES-Vet, 2007; Srinivasan et al., 2007), 0 to 5% for ceftiofur (FINRES-Vet, 2007; Erskine et al., 2002b), and 7 to 21% for ampicillin (Lanz et al., 2003; Lehtolainen et al., 2003) across various studies. Resistance proportions in *Klebsiella* species isolates also varied greatly from 7 to 33% for tetracycline (Bengtsson et al., 2009; Erskine et al., 2002b), 9 to 12% for sulfisoxazole/sulfamethoxazole (Bengtsson et al., 2009; Makovec and Ruegg, 2003) and 0 to 14% for ceftiofur (Saini et al., 2011a; Erskine et al., 2002b) across studies.

Many studies have determined antimicrobial susceptibility of bovine mastitis coliforms (Lanz et al., 2003; Lehtolainen et al., 2003; Makovec and Ruegg, 2003; Srinivasan et al., 2007; Bengtsson et al., 2009), and some studies have evaluated the dynamics of AMR in coliforms isolated from the feces of young dairy calves, dairy cattle, beef cattle and swine (Berge et al., 2005; Akwar et al., 2008; Checkley et al., 2008; Berge et al., 2010). Unfortunately, there is little information on the impact of therapeutic and prophylactic AMU on AMR in bovine mastitis *E. coli* and *Klebsiella* species isolates (Srinivasan et al., 2007). Therefore, it seems prudent to assess and evaluate relationship between AMU and AMR in bovine mastitis coliforms.

The objective of the present study was to determine a herd-level association between AMU and AMR in *E. coli* and *Klebsiella* species isolated from intramammary infections (IMI) and (sub) clinical bovine mastitis cases on Canadian dairy farms.

6.3 Materials and Methods

6.3.1 Herd Selection

Data for this study originated from the National Cohort of Dairy Farms of the Canadian Bovine Mastitis Research Network (CBMRN), which consisted of 91 commercial dairy farms located in four regions across Canada (Alberta, Ontario Québec, and the Maritime Provinces [Prince Edward Island, Nova Scotia, and New Brunswick]). Herd selection criteria for the present study have been described by (Reyher et al., 2011). In short, dairy herds were selected to replicate the regional proportion of free-stall systems to within 15 percentage points and to be uniformly distributed among three strata of the most recent 12-month bulk tank SCC average (≤ 150,000 cells/mL, > 150,000 and ≤ 300,000 cells/mL, > 300,000 cells/mL); herds with three times milking schedule per day and herds with less than 80% (or less than 15) Holstein lactating and dry cows at the time of enrolment were excluded. Further, eligible dairy herds must have been participating in a DHI data collection program. Eligible dairy farms were identified and contacted by the regional center coordinators. Written consent to participate in the research cohort was obtained. Two dairy herds dropped out of the study at the very beginning leaving 89 herds in the study. An investigator and technicians in each coordinating center were responsible for the data collection activities related to remaining farms located in that center's area.

6.3.2 Antimicrobial Use Data Collection Methodology

Antimicrobial use data collection has been described by Saini et al. (2011b). In short, AMU data were collected from February 2007 until December 2008. Forty-liter receptacles were placed on participating farms for collecting data for AMU. These receptacles were placed near the drug storage area, in the milking parlour or any place near where the treatments

were normally given. Producers, farm workers and other farm personnel were instructed to deposit the empty containers of all drugs used by them or the veterinarian for treatment in calves, heifers and adult cows (dry cows and lactating cows) into these receptacles. Farms were visited at least once per month. The research technicians emptied the receptacles, counted the empty drug bottles and recorded the inventory in the drug tally sheets at the dairy farm.

Antimicrobial use data were quantified in units of animal defined-daily doses (ADD). The ADD (g/day) was defined as the average daily on-label dosage multiplied by the approximate weight of an adult dairy cow (BW=600 kg) (Jensen et al., 2004) and was based on the Canadian compendium of veterinary products. Further, antimicrobial drug use rate (ADUR) was defined as number of ADD used on a farm per 1,000 cows (milking and dry) per day. ADUR was the herd-level estimate of AMU (Table 6.1).

6.3.3 Sampling and Bacterial Culturing

Sampling and bacterial culturing of isolates has been described elsewhere (Reyher et al., 2011). In short, three different sets of milk samples were collected. The first set included milk samples from clinical mastitis cases. All producer-diagnosed clinical mastitis cases were sampled at the time of diagnosis and then re-sampled twice at two-week intervals. The second set was from milk samples from nonclinical lactating cows; a sub-sample of 15 fresh and lactating cows was selected per farm. They were aseptically sampled and re-sampled once every 3 weeks for a total of three samplings in the winter of 2007; another sub-sample of 15 lactating cows was sampled once a week for 7 weeks in the summer of 2007.

The third set of milk samples were collected from another selected group of 15 cows that were expected to remain in the herd until at least 2 weeks after calving. A sub-sample was aseptically sampled before dry-off and after calving in 2007 and this continued in 2008 as well. Quarter and composite milk samples were collected. Samples were frozen at -20^0C and shipped to the regional CBMRN laboratory where bacterial culturing and identification of the milk samples was done as per National Mastitis Council guidelines (Hogan et al., 1999).

Because multiple isolates could be coming from a single cow, it was decided to include only one isolate per quarter. Clinical mastitis was defined as an inflammation of the udder leading to occurrence of flakes, clots or other gross alterations in milk. Subclinical mastitis was defined as SCC >200,000 cells/mL from a cow without clinical signs of mastitis, whereas IMI was defined as a culture-positive sample (Reyher et al., 2011).

6.3.4 MIC Determination

Antimicrobial susceptibility testing of bovine mastitis coliforms have been described elsewhere (Saini et al., 2011a). Minimum inhibitory concentrations (MIC) of these isolates were determined using the Sensititre® microdilution system (TREK Diagnostic Systems Inc., Cleveland, Ohio). NARMS Gram-negative plate containing amikacin, gentamicin, kanamycin, streptomycin, ampicillin, amoxicillin-clavulanic acid combination (AMXCLA), cefoxitin, ceftriaxone, ceftiofur, chloramphenicol, ciprofloxacin, nalidixic acid, sulfisoxazole, trimethoprim-sulfamethoxazole combination (TMPS) and tetracycline was used (Varga et al., 2008).

6.3.5 Statistical Analyses

Antimicrobial use data were entered into a customized database (Microsoft Office Access 2006, Microsoft Corporation, Redmond, Washington, USA). A random sample of the drug tally sheets (25%) was checked manually to detect errors in data entry. Data analyses were performed using Intercooled Stata®11.1 (Intercooled Stata for Macintosh, version 11.1, Stata Corporation, College Station, TX).

Minimum inhibitory concentration was defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate. In case of an antimicrobial combination such as TMPS, the MIC of the first agent (trimethoprim) was reported as the MIC for the combination. The isolates were also categorized as sensitive, intermediate and resistant on the basis of CLSI based MIC breakpoints (2008). Intermediate isolates were combined with resistant isolates for the sake of statistical analysis. For analytical purposes, the unit of analysis was resistance at the herd-level (0: number of resistant isolates at a farm = 0; and 1: number of resistant isolates at a farm $\geq$ 1). Herd-level prevalence percentage of AMR was calculated and defined as percentage of total number of herds with AMR isolates divided by total number of herds sampled.

All independent variables (antimicrobial use and other herd-level factors such as region, herd average milk production, barn type, herd average SCC, herd average parity, average herd size, number of isolates sampled at a farm) were screened based on descriptive statistics (means, variances and percentiles for continuous variables, and frequency tabulations for categorical variables) so as to exclude variables that had little variability (Dohoo et al., 2009). Subsequently, these independent variables were screened for

univariate associations using Likelihood Ratio Test Statistic (Hosmer and Lemeshow, 2000). Variables significant at $P \leq 0.25$ were eligible for inclusion in the multivariable logistic regression models. Using backward elimination, variables were retained in model only if significant at $P \leq 0.05$ unless exclusion resulted in significant change in deviance. Thereafter, any variables not selected for the original multivariable model were added back into the model in order to identify variables that were not significantly associated with the herd-level outcome, but made an important contribution in the presence of other variables (distorter variables). Barn type, region, average herd size, and average herd parity were considered *a priori* as potential confounders. Variables as average herd SCC, herd average milk production per cow, average herd size and average herd parity were centered at their respective lowest values for sensible interpretation of the intercept value.

The assumption of linearity in the logit of herd-level AMR outcome for continuous variables was evaluated graphically using lowess smoother scatter plots (Dohoo et al., 2009) and then by using fractional polynomials command in Intercooled Stata®11.1. Two-way interaction terms were added one at a time to the main effects model and retained at $P \leq 0.05$ unless exclusion resulted in significant change in deviance. Robust standard errors were used to control for clustering of farms within regions. In case of nominal variables such as region, the baseline/referent level was selected as the one with sufficiently large sample size (Dohoo et al., 2009).

Multivariable logistic regression models were built for the following AMR outcomes in *E. coli*: tetracycline, ampicillin, ceftiofur, chloramphenicol, TMPS and streptomycin. In case of *Klebsiella* species isolates, a logistic regressions model was only built for tetracycline.

No model could be built for ampicillin, as *Klebsiella* species are intrinsically resistant. Only two and three farms had TMPS and chloramphenicol resistant *Klebsiella* species isolates, respectively; low prevalence of herd-level resistance, therefore, prevented model building for these antimicrobials. Further, variation in streptomycin resistance between barn types within four regions was also very small to prevent model building for *Klebsiella* species isolates.

No models could be built for cefoxitin, ceftriaxone, AMXCLA, amikacin, kanamycin and nalidixic acid resistance outcomes in *E. coli* and *Klebsiella* species isolates, as these antimicrobials were not used on Canadian dairy farms. Additionally, because gentamicin, enrofloxacin and sulfonamides were rarely used on study farms, models could not be built for gentamicin, ciprofloxacin and sulfisoxazole in *E. coli* and *Klebsiella* species isolates as well.

6.4 Results

6.4.1 Antimicrobial Resistance Proportions

Minimum inhibitory concentration values were determined for 394 *E. coli* isolates that came from 394 quarters of 353 cows on 76 dairy farms. Total number of isolates resistant to one or more antimicrobials was 70 or a prevalence of 17.7% (95% CI: 14.1 to 21.9%). Total number of isolates resistant to an antimicrobial ranged from 0 to six per farm. Herd-level AMR prevalence ranged from 0% for ceftriaxone and ciprofloxacin to 38.1% for tetracycline (Table 6.2).

In case of *Klebsiella* species, MIC values were determined for 139 isolates that came from

139 quarters of 114 cows on 37 dairy farms. Fifty-one isolates or 36.6% (95% CI: 28.6 to 45.2%) were resistant to one or more antimicrobials. Total number of isolates resistant to an antimicrobial ranged from 0 to one per farm. Herd-level AMR prevalence ranged from 0% for amikacin, ceftiofur, ceftriaxone, ciprofloxacin and nalidixic acid to 24.3% for tetracycline (Table 6.3).

6.4.2 Multivariable Analyses

The odds for herds having at least one tetracycline-resistant *E. coli* isolate increased with an increasing use of systemically administered tetracycline on farms using tetracycline and penicillin (Table 6.4). Herd-level odds of isolating at least one ampicillin-resistant *E. coli* isolate increased with an increased use of systemically administered penicillin and ceftiofur; the use of systemically administered tetracycline was, however, associated with decreasing odds. Dairy herds in Alberta had significantly higher odds for having at least one tetracycline and ampicillin resistant *E. coli* isolate than dairy herds in Québec. Herds with tie-stall barns had significantly higher odds of having at least one ampicillin-resistant *E. coli* isolate than herds with free-stall barns.

The use of penicillin combinations containing dihydrostreptomycin administered intramammary for clinical mastitis treatment, penicillin-novobiocin combinations administered for dry cow therapy and systemically administered penicillin was positively associated with streptomycin resistance in *E. coli* isolates at the herd-level (Table 6.5). The odds decreased with an increasing use of penicillin combinations administered intramammary for clinical mastitis treatment on farms with larger herd size as compared to farms with smaller herd size. Dairy herds in Ontario had significantly lower odds of having

at least one streptomycin-resistant *E. coli* isolate than dairy herds in Québec.

The odds for herds having at least one TMPS-resistant *E. coli* isolate at a farm increased significantly with an increased use of systemically administered TMPS and ceftiofur, and penicillin combinations administered intramammary for clinical mastitis treatment; however, the use of pirlimycin administered intramammary for clinical mastitis treatment was associated with decreasing odds of TMPS resistance in *E. coli* isolates (Table 6.5). Dairy herds in Alberta had significantly higher odds of having at least one TMPS-resistant *E. coli* isolate than dairy herds in Québec.

Herds with tie-stall barns had significantly higher odds of having at least one ceftiofur-resistant *E. coli* isolate than herds with free-stall barns (Table 6.6). Dairy herds in Alberta had significantly lower odds of having at least one ampicillin-resistant *E. coli* isolate than dairy herds in Ontario.

The use of systemically administered florfenicol and tetracycline was significantly associated with decreasing odds of having at least one chloramphenicol-resistant *E. coli* isolate on a farm; however, the odds increased significantly with an increase in the use of systemically administered penicillin (Table 6.7). The odds increased with an increase in average herd size. Dairy herds in Alberta and Ontario had significantly higher odds of having at least one chloramphenicol-resistant *E. coli* isolate than dairy herds in Québec.

The odds for herds having at least one tetracycline-resistant *Klebsiella* species isolate increased with an increasing use of systemically administered tetracycline at a farm (Table

6.7).

6.5 Discussion

This is the first time that a Canada-wide prospective study was conducted to determine herd-level risk factors of AMR in Gram-negative bovine mastitis pathogens. In addition to AMU, data were collected on herd-level variables such as SCC, milk production, barn type, herd size and geographical region that could potentially explain variation in AMR at the herd-level.

Direct effects of AMU on AMR were observed for some antimicrobials e.g. the use of systemically administered TMP-sulfadoxine combination was positively associated with herd-level TMPS resistance. Intramuscular injection of TMP-sulfadoxine is commonly recommended in cattle; however, it leads to poor udder penetration due to low bioavailability (Prescott, 2006b), and hence could potentially select TMPS resistant strains.

Potential co-selection was observed as well. For example, the use of systemically administered ceftiofur and intramammary administered penicillin combination containing dihydrostreptomycin was positively associated with TMPS resistance. Multi-drug resistance to ampicillin, ceftiofur, streptomycin and TMPS has been observed in bovine mastitis *E. coli* isolates (Saini et al., 2011a). Class I integrons containing ß-lactam, streptomycin and TMP resistance encoding genes have been isolated from multi-drug resistant *E. coli* recovered from cattle and environment (including calves, milk filters, overshoes, water sampled, swabs of the calf pen and feeding bucket) on 21 dairy farms in Ireland (Karczmarczyk et al., 2011). The odds of streptomycin resistance in *E. coli* isolates

increased with an increase in the use of intramammary administered penicillin combination containing dihydrostreptomycin, intramammary penicillin-novobiocin combination and systemically administered penicillin. Intramammary penicillin combinations containing dihydrostreptomycin were most commonly used for clinical mastitis treatment on Canadian dairy farms (Saini et al., 2011b). In terms of potency, spectrum of activity and stability to plasmid mediated enzymatic inactivation, streptomycin is least active among aminoglycosides. Streptomycin / dihydrostreptomycin are synergistic with penicillin in case of Gram-positive bacteria; however, synergism is usually absent in Gram-negative bacteria (Dowling, 2006). Further, Enterobacteriaceae are intrinsically resistant to penicillin, and the mechanisms of resistance to aminoglycosides are different from those of penicillins, therefore these associations appear to be biologically implausible. However, it is quite likely that when dihydrostreptomycin-containing penicillin combinations are used, only the dihydrostreptomycin component is associated with streptomycin resistance, and penicillin has no effect *per se*. Unfortunately, it is not possible to attribute streptomycin resistance to the use of dihydrostreptomycin alone due to its presence with penicillin in a combination product.

Cross-resistance to some antimicrobials was also observed. For example, resistance to ampicillin in *E. coli* isolates was positively associated with the use of systemically administered ceftiofur and penicillin. Resistance to aminopenicillins as ampicillin is usually mediated through the production of ß-lactamases. Penicillins are predominantly ionized in plasma, are less lipid soluble and cross biological membranes poorly; the concentration in milk is about one-fifth of that in serum. Ceftiofur is also poorly distributed in udder due to high plasma protein binding upon systemic administration (Prescott, 2006a). Therefore, it

seems unlikely, that sub-therapeutic concentrations of penicillin and ceftiofur are achieved in the udder upon systemic administration that may cause selection of ampicillin resistant *E. coli* strains in the udder. In fact, the majority of coliforms are from the gut that are shed into the cow's environment and infect udder via the teat canal (Kaipainen et al., 2002; Lehtolainen et al., 2003). It is quite plausible, that selection of AMR occurred inside the gut environment due to AMU in non-mastitis conditions.

Interestingly, the use of certain antimicrobials was negatively associated with AMR in *E. coli*. For example, on dairy farms using only systemically administered tetracycline, the odds for a herd having at least one tetracycline-resistant *E. coli* isolate decreased with an increase in the use of systemically administered tetracycline. Tetracycline use is known to induce the expression of tetracycline resistance via efflux mechanism encoded by *tet* gene in Gram-negative bacteria (Poyart, 2010). Negative associations between AMU and AMR seem to be implausible. These associations could be spurious or due to certain factors that are yet to be identified (Akwar et al., 2008). However, on dairy farms using tetracycline and penicillin, the odds of isolating at least one tetracycline resistant *E. coli* isolate increased with an increase in the use of systemically administered tetracycline thereby indicating an interaction between these two antimicrobials, although systemically administered penicillin was not associated with tetracycline resistance.

In general, average herd parity and barn type tended to be associated with AMR in *E. coli* isolates. Increase in parity indicates an increase in age and therefore increased exposure to antimicrobials and udder pathogens. Subsequent selection pressure could potentially lead to AMR in udder pathogens or changes in the distribution of resistant strains such that

resistant strains become more prevalent in older cows. Herds with tie-stall barns had higher odds for having at least one resistant *E. coli* isolate than herds with free-stall barns. Barn type potentially impacts managemental practices employed at a farm that might be associated with AMR in bovine mastitis pathogens (Kirk et al., 2005). For example, addition of organic or inorganic amendments in bedding may potentially cause an increase or decrease in growth of microorganisms thereby leading to changes in bacterial distributions to those more intrinsically resistant, and hence altering the AMR patterns (Kirk et al., 2005). Increase in interval between grooming and replacement of bedding material was positively associated with AMR. Wet udders had higher odds of AMR than dry udders. In general, dairy practices could potentially be modifiable determinants of AMR in environmental mastitis causing bacteria.

Differences in herd-level prevalence of AMR in bovine mastitis coliforms were observed between regions. Different subpopulations of bacteria with varying antimicrobial susceptibilities exist in different regions (Erskine et al., 2003; Kirk et al., 2005). A region may be a surrogate for management-related differences due to environment, geography, weather, and resources availability. For example, multidrug resistance in fecal *E. coli* isolates collected from cattle (pre-weaned calves on calf ranches, steers on feedlots, dairy and beef cows) were not directly associated with AMU but with geographic region, animal age and purpose (beef versus dairy)(Berge et al., 2010).

Interestingly, some biologically unreasonable associations between AMU and AMR in bovine mastitis coliforms have been observed in this study. However, it is noteworthy that the unit of analysis was farm and not an individual cow. Secondly, data was collected on

herd-level use of antimicrobials; individual cow level AMU data being unavailable, it will be a fallacy to make inferences from this study at individual cow level. Antimicrobial use and resistance data was measured concurrently, and therefore is not reflective of the past AMU that might have a bigger impact on AMR in these isolates. Such group level studies are exploratory in nature as there is no direct measure of the exposure of interest due to measurement constraints at the individual level (Dohoo et al., 2009). These studies are primarily hypothesis generating in nature.

Antimicrobial resistance is potentially a herd-level phenomenon as farm ecosystems provide an ideal environment for emergence, amplification, and dissemination of resistant bacteria/determinants (Acar and Moulin, 2006). In case of bovine mastitis coliforms, it is hypothesized that selection of AMR occurs outside bovine udder in the dairy farm soil due to presence of antimicrobials or AMR bacteria in the feces and urine of animals that accumulate in a dairy farm environment (Burgos et al., 2005; Hammad et al., 2008). In general, pathogens and commensal bacteria are subjected to antimicrobial treatments in a dairy farm environment, and the subsequent selection pressure might favor selection and dissemination of resistant strains (Silbergeld et al., 2008; Tikofsky et al., 2003). Interventions for managing AMR in bacteria should focus at the herd-level and not individual cow level (Tragesser et al., 2006). Various studies have confirmed that AMR in bacteria is not a simple outcome of AMU, and data should be collected on non-antimicrobial use aspects impacting AMR (e.g. dairy practices related to hygiene, nutrition, cow-stress) so as to understand the ecology of resistance in bacteria (Berge et al., 2005; Berge et al., 2010; Gellin et al., 1989; Kirk et al., 2005; Langlois et al., 1988; Sol et al., 2000).

6.6 Conclusions

Antimicrobial resistance in bovine mastitis coliforms was associated directly and indirectly with the use of antimicrobials commonly administered on Canadian dairy farms. Herds with tie-stall barns had higher odds for having at least one resistant coliform isolate than herds with free-stall barns, and average herd parity was associated with herd-level AMR, although statistically non-significant. Geographical variation in AMR was observed that could not be explained by AMU. In addition to AMU data, information should also be collected on dairy practices related to bedding and udder hygiene impacting AMR in bovine mastitis pathogens.

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Table 6.1: Descriptive statistics of herd-level factors and antimicrobial drug use rate (Animal defined-daily doses (ADD)/1000 cow-days) of various antimicrobial drug classes used across 89 dairy farms in 6 Canadian provinces.

Independent Variables	Herds	Mean	SEM	Min.	Median	Max.
Average herd size	89 (100)	84	4.96	33	66	297
Geometric herd SCC mean (x 1000 cells/mL)	89 (100)	230	9.26	91	220	500
Herd average milk production / cow (kg)	89 (100)	32	0.31	25	32	39
Average herd parity	89 (100)	2.5	0.03	1.5	2.5	3.2
		Antimicrobial drug use rate				
Cephalosporins – 1st Gen.	76 (87)	0.92	0.01	0	0.41	7.07
Cephalosporins – 3rd Gen.	80 (90)	2.57	0.02	0	1.24	7.34
Cephalosporins – All	87 (98)	3.49	0.03	0	2.70	8.94
Penicillins	85 (96)	2.81	0.02	0	2.37	7.20
All ß-lactams	89 (100)	6.30	0.04	0.45	5.01	12.87
Penicillin Combination[1]	84 (94)	2.74	0.04	0	1.65	19.68
Tetracyclines	57 (64)	2.52	0.11	0	0.36	50.89
TMP-sulfadoxine Combination	68 (76)	1.10	0.01	0	0.52	3.96
Lincosamides	52 (58)	1.09	0.03	0	0.04	8.91
Macrolides	31 (35)	0.49	0.01	0	0	5.41

Phenicols	29 (33)	0.20	0.004	0	0	1.21
Aminoglycosides	10 (11)	0.09	0.004	0	0	1.28
Ionophores	4 (5)	0.17	0.01	0	0	3.79
Fluoroquinolones	4 (5)	0.004	0.0003	0	0	0.15
Sulfonamides	2 (2)	0.003	0.0002	0	0	0.08
Lincomycin-Spectinomycin	1 (1)	1.10	0.14	0	0	89.61

[1]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

Table 6.2: Descriptive statistics of herd-level antimicrobial resistance outcomes in *Escherichia coli* (n=394) isolated from bovine mastitis cases on 76 dairy farms across 6 provinces in Canada.

Resistance Outcome	Herds[1] (%)	Isolates[2]	Mean[3]	SEM	Min.	Median	Max.
Amikacin	2 (2.6)	2	0.02	0.01	0	0	1
Ampicillin	24 (31.5)	38	0.50	0.09	0	0	3
Amoxi-CLA[4]	7 (9.2)	13	0.17	0.06	0	0	3
Cefoxitin	11 (14.4)	16	0.21	0.07	0	0	5
Ceftriaxone	0	0	--	--	--	--	--
Ceftiofur	4 (5.2)	5	0.06	0.03	0	0	2
Chloramphenicol	10 (13.1)	16	0.21	0.07	0	0	3
Gentamicin	2 (2.6)	2	0.02	0.01	0	0	1
Kanamycin	13 (17.1)	18	0.23	0.06	0	0	3
Streptomycin	24 (31.5)	34	0.44	0.09	0	0	4
Ciprofloxacin	0	0	--	--	--	--	--
Nalidixic acid	1 (1.3)	1	0.01	0.01	0	0	1
Sulfisoxazole	21 (27.6)	36	0.47	0.10	0	0	4
TMP-sulfa[2]	14 (18.4)	22	0.28	0.08	0	0	3
Tetracycline	29 (38.1)	57	0.75	0.15	0	0	6

[1]Number of herds with antimicrobial resistant *Escherichia coli* isolates.

[2]Number of antimicrobial resistant *Escherichia coli* isolates.

[3]Average number of antimicrobial resistant *Escherichia coli* isolates per farm.

[4]Amoxicillin – clavulanic acid combination.

Table 6.3: Descriptive statistics of herd-level antimicrobial resistance outcomes in *Klebsiella* species (n=139) isolated from bovine mastitis cases on 37 dairy farms across 6 provinces in Canada.

Resistance Outcome	Herds[1] (%)	Isolates[2]	Mean[3]	SEM	Min.	Median	Max.
Amikacin	0	0	--	--	--	--	--
Ampicillin			Intrinsic resistance				
Amoxi-CLA[4]	4 (10.8)	9	0.10	0.05	0	0	1
Cefoxitin	5 (13.5)	10	0.13	0.05	0	0	1
Ceftriaxone	0	0	--	--	--	--	--
Ceftiofur	0	0	--	--	--	--	--
Chloramphenicol	3 (8.1)	4	0.08	0.04	0	0	1
Gentamicin	1 (2.7)	2	0.02	0.02	0	0	1
Kanamycin	6 (16.2)	9	0.16	0.06	0	0	1
Streptomycin	7 (18.9)	21	0.18	0.06	0	0	1
Ciprofloxacin	0	0	--	--	--	--	--
Nalidixic acid	0	0	--	--	--	--	--
Sulfisoxazole	8 (21.6)	17	0.21	0.06	0	0	1
TMP-sulfa[2]	2 (5.4)	3	0.05	0.03	0	0	1
Tetracycline	9 (24.3)	28	0.24	0.04	0	0	1

[1]Number of herds with antimicrobial resistant *Klebsiella* species isolates.

[2]Number of antimicrobial resistant *Klebsiella* species isolates.

[3]Average number of antimicrobial resistant *Klebsiella* species isolates per farm.

[4]Amoxicillin – clavulanic acid combination.

Table 6.4: Final logistic regression models depicting herd-level association between antimicrobial use and antimicrobial resistance to tetracycline and ampicillin in *Escherichia coli* (n=394) isolated from bovine mastitis cases on 76 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Tetracycline	Tetracycline use (systemic)	0.04	0.06	0.02	0.003 to 0.62
	Penicillin use (systemic)	1.99	1.34	0.30	0.53 to 7.46
	Tetracycline use (systemic) * Penicillin use (systemic)**	3.61	2.35	0.049	1.006 to 12.95
	Average herd size	1.16	0.07	0.014	1.03 to 1.31
	Average herd parity	1.26	0.73	0.68	0.40 to3.92
	Barn type (0 - free stall, 1 - tie stall)	1.54	1.12	0.55	0.36 to 6.46
	Region:				
	Québec (baseline)	1.00			
	Ontario	1.01	0.17	0.94	0.72 to 1.42
	Alberta	2.54	0.98	0.01	1.19 to 5.42
	Maritimes	1.92	1.11	0.25	0.62 to 5.98
Ampicillin	Penicillin Combination[1] use (Lactating cow therapy)	1.42	0.28	0.079	0.95 to 2.12
	Ceftiofur use (systemic)	21.33	13.06	< 0.01	6.42 to 70.81
	Penicillin use (systemic)	3.73	2.44	0.04	1.03 to 13.50
	Tetracycline use (systemic)	0.01	0.02	0.01	0.0007 to 0.42
	Herd average milk production	0.52	0.09	< 0.01	0.36 to 0.75
	Tetracycline use (systemic) * Herd av. Milk production**	1.51	0.35	0.07	0.95 to 2.40
	Average herd size	1.009	0.05	0.86	0.90 to 1.12
	Average herd parity	1.58	0.75	0.33	0.62 to 4.05
	Barn type (0 - free stall, 1 - tie stall)	14.15	18.31	0.04	1.12 to 178.79
	Region:				

Québec (baseline)	1.00			
Ontario	0.76	0.34	0.55	0.31 to 1.86
Alberta	5.48	1.18	< 0.01	3.59 to 8.38
Maritimes	0.61	0.34	0.38	0.20 to 1.83

[1]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

** Interaction between explanatory variables.

Table 6.5: Final logistic regression models depicting herd-level association between antimicrobial use and antimicrobial resistance to streptomycin and Trimethoprim-sulfamethoxazole (TMPS) combination in *Escherichia coli* (n=394) isolated from bovine mastitis cases on 76 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Streptomycin	Penicillin Combination[1] use (Lactating cow therapy)	2.26	0.80	0.02	1.13 to 4.54
	Penicillin-novobiocin use (dry cow therapy)	1.75	0.37	< 0.01	1.14 to 2.66
	Penicillin use (systemic)	3.31	0.62	< 0.01	2.29 to 4.79
	Tetracycline use (systemic)	0.29	0.22	0.11	0.06 to 1.33
	Average herd size	1.45	0.30	0.07	0.96 to 2.19
	Penicillin combination use * average herd size**	0.87	0.03	< 0.01	0.80 to 0.95
	Average herd parity	1.29	0.86	0.69	0.35 to 4.79
	Barn type (0-free stall, 1- tie stall)	0.89	0.88	0.91	0.13 to 6.17
	Region:				
	Québec (baseline)	1.00			
	Ontario	0.64	0.07	< 0.01	0.52 to 0.79
	Alberta	2.71	2.09	0.19	0.59 to 12.32
	Maritimes	1.88	1.72	0.48	0.31 to 11.32
TMPS	TMPS use (systemic)	1.68	0.45	0.05	0.99 to 2.86
	Ceftiofur use (systemic)	31.08	21.02	< 0.01	8.25 to 117.01
	Penicillin Combination[1] use (Lactating cow therapy)	1.53	0.16	< 0.01	1.24 to 1.89
	Pirlimycin use (Lactating cow therapy)	0.44	0.05	< 0.01	0.35 to 0.55
	Average herd size	0.96	0.08	0.72	0.81 to 1.14
	Average herd parity	0.79	0.47	0.69	0.24 to 2.53
	Barn type (0-free stall, 1- tie stall)	2.27	1.78	0.29	0.48 to 10.58

Region:				
Québec (baseline)	1.00			
Ontario	0.63	0.15	0.06	0.39 to 1.02
Alberta	1.89	0.39	< 0.01	1.26 to 2.85
Maritimes	2.00	0.78	0.07	0.92 to 4.32

[1]Intramammary preparation containing Penicillin G Procaine/Dihydrostreptomycin sulfate/Novobiocin sodium/Polymyxin B sulfate.

** Interaction between explanatory variables.

Table 6.6: Final logistic regression models depicting herd-level association between antimicrobial use and antimicrobial resistance to ceftiofur and chloramphenicol in *Escherichia coli* (n=394) isolated from bovine mastitis cases on 76 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Ceftiofur	Ceftiofur use (systemic)	0.96	0.11	0.76	0.77 to 1.20
	Average herd size	0.95	0.07	0.57	0.82 to 1.11
	Average herd parity	1.52	1.34	0.63	0.26 to 8.63
	Barn type (0-free stall, 1- tie stall)	10.06	6.72	< 0.01	2.71 to 37.28
	Region:				
	Québec	Omitted due to absence of resistance			
	Ontario (baseline)	1.00			
	Alberta	0.13	0.007	< 0.01	0.12 to 0.15
	Maritimes	Omitted due to absence of resistance			
Chloramphenicol	Florfenicol use (systemic)	0.08	0.04	< 0.01	0.02 to 0.26
	Penicillin use (systemic)	3.04	1.21	0.005	1.39 to 6.66
	Tetracycline use (systemic)	0.008	0.01	0.003	0.0003 to 0.19
	Average herd size	1.21	0.07	0.001	1.08 to 1.36
	Average herd parity	0.87	0.22	0.62	0.52 to 1.46
	Barn type (0-free stall, 1- tie stall)	1.16	0.78	0.81	0.31 to 4.35
	Region:				
	Québec (baseline)	1.00			
	Ontario	2.77	0.39	< 0.01	2.09 to 3.67
	Alberta	20.27	4.65	< 0.01	12.92 to 31.79
	Maritimes	1.93	0.74	0.09	0.90 to 4.13

Table 6.7: Final logistic regression model depicting herd-level association between tetracycline use and tetracycline resistance in *Klebsiella* species isolates (n=139) from bovine mastitis cases on 37 dairy farms in 6 Canadian provinces.

Resistance Outcome	Variables	Odds ratio	Robust SE	*P*- value	95% CI
Tetracycline	Tetracycline use (systemic)	2.59	1.07	0.02	1.14 to 5.86
	Average herd size	0.97	0.03	0.38	0.90 to 1.03
	Average herd parity	4.20	3.82	0.11	0.70 to 24.90
	Barn type (0 - free stall, 1 - tie stall)	0.20	0.45	0.47	0.002 to 15.89
	Region:				
	Maritimes (baseline)	1.00			
	Ontario	2.37	1.19	0.08	0.88 to 6.37
	Alberta	22.92	41.54	0.08	0.65 to 799.98
	Québec	Omitted due to absence of resistance			

Chapter Seven: Conclusions and Future Perspectives

The general objectives of this thesis included: a) Validating methods commonly employed for antimicrobial susceptibility testing of bovine clinical mastitis pathogens, b) Determining qualitative and quantitative aspects of antimicrobial drug utilization on Canadian dairy farms, c) Antimicrobial susceptibility testing of *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella* species pathogens isolated from intramammary infections and (sub) clinical mastitis cases, and d) Assessing and evaluating if a herd-level association between antimicrobial use and resistance exists in common mastitis pathogens.

Bacteria isolated from different animal species or even different sites of infection might vary in growth; such differences could impact antimicrobial susceptibility results. Sensititre® MIC susceptibility system and the agar disk diffusion method was therefore validated with manual broth microdilution test method for clinical mastitis pathogens isolated from dairy cattle. The agar disk diffusion method and the Sensititre® MIC susceptibility system had a moderate to high diagnostic accuracy for most antimicrobial-isolate species combinations. As part of future research, it will be interesting to determine if similar results are also observed for isolates from intramammary infections and subclinical mastitis cases as well.

Even though the method of auditing empty antimicrobial containers was producer friendly and circumvented issues of producer non-compliance observed in antimicrobial drug utilization studies, it fails to account for actual usage including off-label use at the individual cow-level. Data on dose and duration of actual treatment administered are vital

for determining selection pressure imposed by antimicrobial treatment on bacteria in an animal. For example, under-dosing could facilitate acquiring of resistance mechanisms in surviving mastitis pathogens whereas over-dosing could result in faster rate of resistance development such as through the ‘Eagle-effect’ as observed for ß-lactams. The concept of prescribed-daily dose is therefore superior to defined-daily dose. The prescribed-daily doses may be calculated from prescriptions, information from veterinarian’s invoices or producer-written treatment records. However, producer compliance in filling complete and accurate information on health records is a potential challenge. In future, electronic means of capturing antimicrobial use and health data at the individual cow-level such as hand-held computers for producers should be relied upon to facilitate producer compliance. Discordance between antimicrobial use data collected at an individual cow-level and herd-level should also be determined for assessing measurement bias in such studies.

One isolate per quarter was selected for antimicrobial susceptibility testing. It was done to ensure statistical independence between isolates presuming that emergence of antimicrobial resistance in an isolate in a quarter might impact antimicrobial resistance in another isolate in the same quarter. This seems unlikely because environment inside the udder is dynamic compared to gastro-intestinal tract where reservoirs of resistance determinants do not exist. In future studies, one isolate per cow should be selected for antimicrobial susceptibility testing. The present study could not assess a temporal association between antimicrobial use and resistance in bovine mastitis pathogens; such associations can only be assessed in a case-control or cohort study. It is therefore recommended, that follow-up studies should be carried out at the individual cow-level in the future.

Antimicrobial resistance prevalence was uncommon in *S. aureus*, *E. coli*, and *Klebsiella* species isolates. Resistance to antimicrobials of very high importance to human medicine was rare in bovine mastitis pathogens. The study results suggest a low risk of transmission of antimicrobial resistant bacteria from milk or milk products to human populations in Canada. However, monitoring of antimicrobial resistance in udder pathogens should remain ongoing for collecting information concerning emergence and trends of antimicrobial resistance in bacterial populations.

The use of antimicrobials in food-animal production systems remains a contentious issue. Among antimicrobials of very high importance in human medicine, fluoroquinolones were rarely used, whereas third-generation cephalosporins and penicillin combinations containing colistin were used very frequently on Canadian dairy farms. The author believes that there is room for conservative use of antimicrobials in dairy cattle. For example, most of the commercial antimicrobials used for dry cow therapy are active against Gram-positive udder pathogens, and have little or no activity against Gram-negative pathogens. Most clinical mastitis cases caused by coliforms can resolve on their own without the use of antimicrobials. In general, knowledge about the role of cow and pathogen factors in therapeutic success of bovine mastitis is important while planning antimicrobial treatment regimen (Barkema et al., 2006). The author believes that the use of antimicrobials will remain an inalienable part of food-animal production system, but prudent (appropriate, justifiable, judicious) use of antimicrobials is vital for containing antimicrobial resistance, and that continuing education of producers is a key step towards conservative use of antimicrobials.

Antimicrobial resistance in bacteria at a farm cannot be solely explained in terms of antimicrobial use. Emergence and dissemination of antimicrobial resistance is a complex phenomenon and depends upon the geographical location, hygiene levels, herd size, and the type of integrated farming that takes place at a farm (Acar and Moulin, 2006). Use of antimicrobials in animals, and antibiotic residues and resistant bacteria in the feces, effluents, wastewater lagoons and soil create a reservoir of antimicrobial resistance determinants that can transfer between different compartments / niches at a farm. Similar observations have been made in this study e.g. barn type, herd size and region was associated with herd-level antimicrobial resistance outcomes. In future, besides antimicrobial use, information should be collected on such farm-level factors that impact the ecology of antimicrobial resistance in bacteria.

7.1 References

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