CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE AMONG CANINE URINARY ISOLATES IN WESTERN CANADA

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By

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ABSTRACT

Background: Urinary tract infections (UTIs) are common in veterinary medicine. *Escherichia coli* (*E. coli*) and *Staphylococcus pseudintermedius* (*S. pseudintermedius*) are the two most common causes of canine UTIs. The emergence of resistance to first line antimicrobials in these pathogens has posed challenges for veterinarians to treat their patients. Surveillance to detect the emergence of antimicrobial resistance (AMR) in companion animal pathogens is lacking in Western Canada. The efficacy of alternative agents for treating canine UTIs is similarly under investigated. The objective of this study was to determine the frequency and characterize the mechanisms of AMR among canine urinary pathogens in Saskatoon, Canada.

Methods: Non duplicate canine urinary isolates (516 *E. coli* and 113 *S. pseudintermedius*) were collected from a diagnostic laboratory between November 1st, 2014 and October 31st, 2018. Susceptibility testing was performed by agar dilution or broth microdilution against 15 antimicrobials belonging to 8 different drug classes and interpreted according to published breakpoints. Molecular characterization of resistance was performed by polymerase chain reaction (PCR) and epidemiological relationships were assessed by multi-locus sequence typing (MLST).

Results: A substantial majority of isolates were susceptible to all antimicrobials tested. There was no significant increase in the frequency of resistance to any of the tested agents during the study period. Among *E. coli*, resistance to ampicillin was most common. Seven *E. coli* and four *S. pseudintermedius* were fosfomycin resistant. Overall, twelve isolates harbored CMY-2 type AmpC β-lactamases, and seven produced CTX-M

type extended spectrum β-lactamases (ESBLs). Of the fosfomycin resistant *E. coli*, one also possessed an CMY-2 type AmpC β-lactamase. Of the fosfomycin resistant *S. pseudintermedius*, three were methicillin resistant. One isolate produced the *aac(6')- Ib-cr* gene. A single isolate belonging to the pandemic lineage ST131 was identified.

Conclusion: Canine urinary *E. coli* in Saskatchewan remain largely susceptible to first line therapies, though resistance, particularly to the aminopenicillins, warrants continued monitoring. Fosfomycin represents a viable alternative option for treating uncomplicated cystitis in canine patients, although continued surveillance is required to identify changes in resistance. This is the first description of *E. coli* ST131 from a companion animal pathogen in Canada.

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LIST OF ABBREVIATIONS

ACC	Ambler class C
ACT	AmpC type
AMP	Ampicillin
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ARESC	Antimicrobial Resistance Epidemiological Survey on Cystitis
AST	Antimicrobial susceptibility testing
AUG	Amoxicillin-clavulanate
AXO	Ceftriaxone
AZI	Azithromycin
BLAST	Basic local alignment search tool
CANWARD	Canadian ward surveillance study
CARD	Comprehensive antimicrobial resistance database
CARSS	Canadian antimicrobial resistance surveillance system
CDC	Centers for Disease Control and Prevention
CGA	Clonal group A
CHL	Chloramphenicol
CIP	Ciprofloxacin,
CIPARS	Canadian integrated program for antimicrobial resistance surveillance
CLI	Clindamycin
CLSI	Clinical Laboratory Standards Institute
CMY	Cephamycin
CTX-M	Cefotaxime hydrolyzing capabilities

DAP	Daptomycin
DHA	Dhahran Hospital
ECO.SENS	European *E. coli* sensitivity survey
ERY	Erythromycin
ESBL	Extended spectrum β-lactamase
ESC	Extended spectrum cephalosporins
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Union committee for antimicrobial susceptibility testing
ExPEC	Extra-intestinal pathogenic *E. coli*
FOS	Fosfomycin
FOX	Cefoxitin
G6P	Glucose-6-phosphate
GEN	Gentamicin,
GLASS	Global antimicrobial resistance surveillance system
IDSA	Infectious diseases society of America
IMP	Imipenem
ISCAID	International society for companion animal infectious disease
KPC	*Klebsiella pneumoniae* carbapenemase
LEVO	Levofloxacin
LZD	Linezolid
MALDI-TOF	Matrix-based assisted laser desorption ionization time-of-flight spectroscopy
MBL	Metallo-β-lactamase
MCR	Mobile colistin resistance
MDR	Multidrug resistant

MER	Meropenem
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MOX	Moxalactam
MRSA	Methicillin resistant *Staphylococcus* aureus
MRSP	Methicillin resistant *Staphylococcus pseudintermedius*
MXF	Moxifloxacin
MYSTIC	Meropenem yearly susceptibility test information collection
NAL	Nalidixic acid
NAUTICA	North America urinary tract infection collaborative alliance
NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo-β-lactamase
NIT	Nitrofurantoin
NSAID	Nonsteroidal anti-inflammatory drugs
OMP	Outer membrane protein
OXA	Oxacillin hydrolyzing capabilities
PCR	Polymerase chain reaction
PDS	Prairie Diagnostic Services
PEN	Penicillin
PMQR	Plasmid mediated quinolone resistance
RIF	Rifampin
SHV	Sulfhydryl variable
SMART	Study for monitoring antimicrobial resistance trends
SNP	Single nucleotide polymorphism
SOX	Sulfisoxazole

STR	Streptomycin
SXT	Trimethoprim-sulfamethoxazole
SYN	Quinupristin/dalfopristin
TEM	Temoneira
TET	Tetracycline
TGC	Tigecycline
TSB	Tryptic soy broth
TSI	Triple sugar iron
UTI	Urinary tract infection
VAN	Vancomycin
VIM	Verona integron-encoded metallo-β-lactamase
VRE	Vancomycin resistant *Enterococci*
WHO	World Health Organization
WGS	Whole genome sequencing
XNL	Ceftiofur

1. LITERATURE REVIEW

1.1 An introduction to antimicrobial resistance

Antimicrobial resistance (AMR) is a normal microbial defense mechanism and many antimicrobial agents were first identified in microorganisms (fungi and bacteria) in nature[1]. In fact, through metagenomic analysis, genes encoding tetracycline, β-lactam and glycopeptide resistance have been found in 30 000-year-old DNA right here in Canada[2]. Since the commercialization of penicillin in 1943, widespread antimicrobial use (AMU) in medicine and agriculture has imposed a selective pressure that has prompted the emergence and spread of resistance[3]. The link between antimicrobial consumption and the emergence and the dissemination of AMR strains has been well documented by epidemiological studies[3]. For example, in Europe, significant intercountry variation exists in the quantity of antimicrobials used to raise similar numbers of animals for human consumption[4]. A comparison of the quantity of antimicrobials consumed and the frequency of resistance in commensal *E. coli* from pigs, poultry and cattle in seven European countries suggests an overall strong positive correlation between AMU and AMR for many drug classes including fluoroquinolones, sulfonamides, aminopenicillins and third generation cephalosporins[5]. What's more, rising concern for the contribution of environmental reservoirs to the AMR problem have been identified recently[6]. Specifically, the contamination of environments, including food and water sources, which are shared between humans and animals are increasingly investigated[6–8].

Social factors also contribute to the AMR problem. Changes in health seeking behaviour create an increasing demand for antimicrobials, while the widespread

availability and lack of regulation in some countries has increased access to antimicrobials[9]. What's more, the development of new agents is plagued by regulatory and financial constraints. While drugs for treating chronic diseases have significant financial return, the restricted use and significant cost associated with bringing antimicrobials to market has eliminated the financial incentive to develop new agents that is reflected in the relatively low number of companies currently cultivating new drugs[10]. Factor in the cost associated with regulatory requirements, including clinical trials and licensing, and the net value of developing a new antimicrobial is projected to be $-50 million, compared to +1 billion for a novel treatment for neuromuscular disease[10].

The social and economic consequences of AMR are equally as staggering. Projections from the European Centre for Disease Prevention and Control suggest that the 400 000 infections due to common multidrug resistant (MDR) infections result in 25 000 deaths annually[11]. An additional 2 million people fall ill and 23 000 die each year due to similar infections in the United States[12]. Furthermore, the health care costs and productivity losses due to these infections in the U.S. are on the order of 20 and 55 billion USD respectively[13,14]. While the development of AMR is rapid, reversing it is comparably slow. Important strategies to combat AMR include surveillance activities and restrictive policies to decrease AMU in humans, animals and agriculture[1].

1.2 The clinical context of canine uropathogens

1.2.1 Dogs as reservoirs for antimicrobial resistance

When an animal takes antimicrobial drugs, its entire microbial community (including zoonotic, commensal and pathogenic bacteria) are exposed to the agent[15]. In fact, it has been shown that fecal *E. coli* from healthy and hospitalized dogs harbour resistance to one or more commonly prescribed antimicrobials, even in the absence of previous therapy[16–20]. Resistant bacteria are similarly isolated from extra intestinal sites. In a retrospective case series assembled from clinical data and laboratory specimens of MDR extra-intestinal *E. coli* from canine patients, the urinary tract accounted for 62% of isolates[21]. While one host may experience no adverse effects, these commensal populations represent an important reservoir for resistance genes that have the potential to cause disease under suitable circumstances. For example, the close contact between humans and their pets has implications for the sharing of resistant commensal and pathogenic bacteria between species, a phenomenon referred to as the Canine Reservoir Hypothesis[19]. Though the exact role of dogs in this zoonotic transmission cycle is poorly understood, examples supporting “strain sharing” between humans and companion animals are numerous in the literature. Among seven unique *E. coli* strains isolated from household members of a women with acute cystitis, the offending UTI strain was extensively shared, in time and space, between five members, including the family dog[22]. The sharing of canine fecal and urinary *E. coli* between dogs and humans has also been demonstrated by the simultaneous isolation of MDR and highly pathogenic strains from hospital workers and canine patients in a clinic environment[23]. This phenomenon is not restricted to *E. coli*. Human epidemic clones of MDR

Enterococci and methicillin resistant *Staphylococcus aureus* (MRSA) have also been isolated from pets in both the hospital and home environment[24–27]. Still others have identified an association between canine exposure to human or hospital environments and colonization with MRSA and *Clostridium difficile*[28]. At the molecular level, MDR Gram-negative pathogens carrying the same resistant genes have been isolated from dogs, cats, horses and people[20,29]. However, the presence of similar genes does not imply the isolation of identical strains. In reality, zoonotic transmission of resistant organisms from companion animals to humans is difficult to prove[30].

1.2.2 Antimicrobial use in canine patients

Canadian data indicates that per year, dog owners are willing to spend almost double that of cat owners for veterinary services[31]. With this changing value of animals in society, more companion animals are receiving antimicrobial treatment. The most common infections for which antimicrobials are prescribed in dogs are skin, ear, respiratory and urogenital infections[30,32]. Concern for the potential of companion animals and livestock to harbour MRSA has led to a significant body of literature describing AMR among canine skin pathogens[33–35]. Urogenital infections remain relatively under investigated in comparison.

Unlike human medicine, veterinarians both prescribe and dispense medication, including antimicrobials, at the individual practice level. As a result, there is no central body collecting data on veterinary prescriptions in Canada[30]. In fact, the only countries effectively tracking AMU in companion animals at a national level are Sweden, France, Finland, Denmark, Norway and the UK[1,30,36–41]. While concern for AMU in food animals

is not new, there are some patterns emerging in the prescribing practices of companion animal veterinarians that warrant attention. In the UK, the small animal veterinary surveillance network (SAVSNET) identified that roughly one third of consults with canine patients results in the prescription of antimicrobials[42]. The most commonly prescribed antimicrobial in this population was the potentiated β-lactam, amoxicillin-clavulanate[42–44]. Among companion animals in the UK, the β-lactam family accounted for three quarters of all antimicrobials prescriptions[42]. Surveillance efforts in Norway indicate similar AMU trends in pet animals. Since its approval for veterinary use in 1994, the proportion of amoxicillin- clavulanate prescriptions, relative to the total number of antimicrobial prescriptions in companion animals in Norway, rose from 1-84% in 2017[39]. The fluoroquinolones are a family of broad spectrum and fully synthetic antimicrobials that were introduced into veterinary use in the 1990s[45]. According to national wholesale records in Denmark, a relatively small number of dogs and cats (1.2 million), consumed the same weight of fluoroquinolones and cephalosporins as food producing animals (including 23 million slaughter pigs, 130 million broiler chickens, and 1.2 million cattle and dairy cows), in 2003, pointing to the potential inappropriate use of these important drug classes among companion animal veterinarians[46].

No matter the motivation, AMU provides a selective pressure for the acquisition of resistance. This link between AMU and the development of resistance is particularly well documented for the fluoroquinolones. Among canine patients hospitalized in an intensive care unit, those that were treated with enrofloxacin were 25.6 times as likely to be colonized by a quinolone-resistant *E. coli* strain compared to animals in which antimicrobial were not used[47]. Similarly, an investigation of canine urinary *E. coli* isolates

in a U.S. teaching hospital revealed a significant increase in enrofloxacin resistant isolates that mirrored an increase in prescriptions the preceding year[48]. Concern about the overuse of certain families of antimicrobials in veterinary medicine, has led to the development of evidence-based consensus statements on disease specific AMU[15,49,50]. Factors guiding prudent use include awareness of the most common offending pathogens and their susceptibility patterns, as well as consideration for safety, and guided where possible by microbial culture and susceptibility testing against clinically relevant drugs[51]. Regulatory bodies in other regions have gone a step further and greatly restricted or banned the veterinary use of certain antimicrobials. In northern Europe, for example, the use of certain fluoroquinolones and third generation cephalosporins is tightly regulated. However, such restrictive policies are often only applied to food producing animals and are not extended to include companion animals[15].

1.2.3 A clinical description of canine urinary tract infections

1.2.3.1 Etiology and pathogenesis

Urinary tract infections (UTIs) affect between 5-27% of dogs that visit a veterinarian in their lifetime, with spayed females being over represented[52,53]. A UTI results from a temporary or permanent break in host defense, which allows microbes to invade, multiply and persist in the urinary tract[52]. Where there is spontaneous colonization of the urinary tract with compatible signs of lower urinary tract disease, a simple or uncomplicated infection exists[54]. While this terminology was previously used to describe infections in patients with no underlying comorbidities, structural, or functional abnormalities of the urinary tract, the most recent guidelines from the International

Society for Companion Animal Infectious Disease (ISCAID) assert that sporadic infections can still occur in these patient populations[54]. Complicated or recurrent infections, on the other hand, describe those that occur concurrently with an underlying condition, such as kidney disease or diabetes, which compromises host defense mechanisms. Complicated infections also encompass those that occur recurrently (three or more infections in a single year); or occur in a comparatively rare patient population; such as male dogs; or location; such as the kidneys or prostate[52]. Interestingly, feline cystitis used to be classified as “complicated” due to the frequent occurrence in senior patients, however, evidence that these infections are more difficult to manage is lacking[54].

Most often, invasion of the urinary tract occurs when microorganisms from the normal flora of the skin or gastrointestinal tract ascend the urethra to cause inflammation of the bladder (cystitis). Colonization of the upper urinary tract, including ureters and kidneys, is possible and is referred to as pyelonephritis[52,55]. However, for the purposes of this review, UTI is synonymous with cystitis and infections of the lower urinary tract.

The most common etiological agent in canine UTI cases is the Gram-negative enteric bacteria *E. coli* belonging to the larger Enterobacteriaceae family. Though estimates vary, *E. coli* represent between one third and one half of positive urine cultures[52,56]. The Gram-positive cocci *Staphylococcus*, *Streptococcus* and *Enterococcus* spp, which normally colonize the canine skin, occur less frequently. Additional pathogens identified in canine UTI cases include the Enterobacteriaceae *Proteus, Klebsiella* and *Enterobacter* spp[57]. The most common offending agents in cases of canine UTIs are summarized in Table 1.1[51,57].

Table 1.1: The ten most frequent pathogens cultured in canine urinary tract infections[57]

Microorganism	Prevalence (%)
E. coli	44.1
Staphylococcus spp.	11.6
Proteus spp.	9.3
Klebsiella spp.	9.1
Enterococcus spp.	8.0
Streptococcus spp.	5.4
Pseudomonas spp.	3.0
Mycoplasma spp.	2.5
Enterobacter spp.	2.3
Providencia spp.	1.0

1.2.3.2 Diagnosis

Clinically, a diagnosis of UTI is made based on a combination of compatible findings in the history, physical exam, and ancillary tests[58]. Clinical signs of cystitis include frequent or painful urination, straining to urinate, urinating blood or inappropriate urination (accidents in the house)[58]. While physical examination is often unremarkable, a patient side visual and microscopic evaluation of urine, known as urinalysis, may reveal elevated protein and red blood cells (hematuria) with or without elevated white blood cells (pyuria) and bacteria (bacteriuria), which are suggestive of infection[58]. However, the submission of a urine sample, obtained by cystocentesis, for aerobic culture and susceptibility testing is required for definitive diagnosis of UTI, to guide antimicrobial prescription and confirm infection[50,58].

1.2.3.2.1 Antimicrobial susceptibility testing

Susceptibility testing involves exposing bacteria to a range of concentrations of a panel of drugs to detect the presence of resistance and to confirm susceptibility to a drug of choice[59]. Both qualitative and quantitative methods exist. For example, disk diffusion is a technique commonly employed in veterinary diagnostic labs. By this method bacteria are designated simply as susceptible, intermediate or resistant[59]. This fact limits the information which may be generated from the results. In contrast, quantitative susceptibility testing methods, which have become standard in medical diagnostic laboratories, involve the inoculation of a standard number of bacteria in broth onto 96 well trays or agar plates containing serial dilutions of various antimicrobials, followed by overnight incubation and the determination of the minimum concentration

that inhibits bacterial growth (known as minimum inhibitory concentration or MIC)[59]. MIC values (measured in μg/mL) can be compared to published breakpoint for each drug-bug combination tested. Bacteria exhibiting MICs above this breakpoint value are defined as clinically resistant. A “susceptible” result indicates that an organism should be readily treated with the corresponding antimicrobial using the normal dosage recommendations for that type of infection and species. Conversely, a “resistant” organism is not anticipated to respond to therapy at the concentration of antimicrobial achieved with normal dosages for the infection under investigation[58]. With these quantitative methods it is possible to detect both reduced susceptibility and clinical resistance. Epidemiological cut-off values have been published, which indicate the highest MIC displayed by a wild type member of a bacterial species, which is devoid of phenotypically detectable acquired resistance. An elevated MIC relative to this value indicates the presence of acquired resistance that is not typical for that bacteria, a fact that is not as readily detected by qualitative susceptibility testing methods. Both agar dilution and broth microdilution techniques have been highly standardized and are available in a number of commercially prepared formats[59]. It is recommended that the diagnostic laboratory perform and interoperate susceptibility testing according to published standards such as those available through the Clinical Laboratory Standards Institute (CLSI) using criteria specific to urinary pathogens where available[58]. These values have taken into account microbiological, pharmacologic and clinical data to synthesize the efficacy of certain drug-bug combinations for the infection under investigation[50].

1.2.3.3 Treatment

Antimicrobials have historically been the mainstay of treatment in cases of sporadic or uncomplicated canine cystitis. However, the most recent ISCAID guidelines suggest that initial treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) may be appropriate with the addition of antimicrobials if clinical signs worsen[54]. Empirical treatment for sporadic cystitis is appropriate only in cases when previous AMU is limited and the causative agent and its most likely susceptibility are predictable[54]. Options include members of the β-lactam class; such as the aminopenicillin amoxicillin, preferably in a formulation without the β-lactamase inhibitor clavulanic acid. The potentiated sulfonamide trimethoprim-sulfamethoxazole (SXT), while frequently used historically, has fallen out of favour due to its significant profile of adverse effects[50,58]. Second tier or alternative therapies with a broader spectrum of activity, such as nitrofurantoin, fluoroquinolones (enrofloxacin, ciprofloxacin), chloramphenicol, doxycycline and fosfomycin should be reserved for cases where laboratory results indicate a lack of susceptibility to empirical treatments[54]. In general, it is considered inappropriate to prescribe third generation cephalosporins, carbapenems, aminoglycosides or fluoroquinolones in cases of sporadic cystitis given their relative importance in treating human infections, coupled with increasing frequencies of resistance[54].

The duration of therapy in UTI cases is a contentious topic in both human and veterinary medicine. Historically, in veterinary patients, therapy is long (7-14 days) compared to similar infections in humans (3-5 days)[58,60]. The limited veterinary literature available indicates that short duration (3 day) therapy with SXT and enrofloxacin were

equivalent to 10-14 days of therapy with amoxicillin-clavulanate or cephalexin in treating uncomplicated cystitis in canine patients[61,62]. In both cases, these studies compared short duration therapy with one agent to long duration therapy with an antimicrobial belonging to a different class. Unfortunately, studies comparing the same drug at different durations of therapy are lacking in veterinary medicine. Despite relatively limited evidence, ISCAID's recommended duration of therapy has been recently updated to 3-5 days[54]. In addition to culture and susceptibility results, these guidelines encourage veterinarians to educate themselves on the pathogen and AMR trends in their area, as well as principles of prudent use, to further guide antimicrobial therapy in UTI cases[15,49,50,54]. Treatment guidelines for canine UTIs are summarized in Table 1.2.

Table 1.2: ISCAID guidelines for the treatment of bacterial cystitis in dogs and cats[54]

Drug	Class	Dose	Comments
Empirical treatment			
Amoxicillin	β-lactam- aminopenicillin	11-15 mg/kg PO q12h	Good first line option. Excreted in the urine predominantly in its active form. Achieves high concentrations in the urine
Amoxicillin-clavulanate	Potentiated β-lactam	12.5-25 mg/kg PO q12h	Good first line choice when regional resistance to amoxicillin is high. No significant evidence for superiority over amoxicillin alone for uncomplicated cystitis. Achieves high concentrations in urine
Trimethoprim-sulfamethoxazole	Potentiated sulfonamide	15-30 mg/kg PO q12h	Good first line option
Second line treatment			
Nitrofurantoin	Nitrofurans	4.4-5 mg/kg PO q8h	Good choice for uncomplicated cystitis particularly when multidrug resistant pathogens is involved
Enrofloxacin/Ciprofloxacin/ Marbofloxacin/Orbifloxacin/ Pradofloxacin	Fluoroquinolone	5-20 mg/kg q24h 25-30 mg/kg PO q24h 2.7-5.5 mg/kg PO q24h 2.5-7.5 mg/kg PO q24 3-5 mg/kg PO q24.	Reserved for multidrug resistant infections
Chloramphenicol	Phenicol	40-50 mg/kg PO q8h	Reserved for multidrug resistant infections
Doxycycline	Tetracycline	5 mg/kg PO q12h	Reserved for infections that are resistant to drugs that achieve high urine concentrations

Fosfomycin	Phosphonic acid derivative	40 mg/kg PO q12h	Reserved for multidrug resistant infections
Not Recommended			
Imipenem/Meropenem	β-lactam-carbapenem	5 mg/kg IV/IM q6-8h 8.5 mg/kg q12 SC or q8 IV	Not recommended for routine use for uncomplicated cystitis
Cefovecin	β-lactam- third generation cephalosporin	8 mg/kg SC once	Not recommended for routine use for uncomplicated cystitis as duration and spectrum are longer than is typically needed. No oral option
Amikacin	Aminoglycoside	15-30 mg/kg IV/IM/SC q24h	Not recommended for routine use for uncomplicated cystitis

1.2.4 Cystitis in human medicine

In the U.S. between 2007 and 2009, antimicrobials were prescribed during 101 million (10%) outpatient physician visits each year[63]. Broad-spectrum agents were used during 61% of these visits with fluoroquinolones (25%), macrolides (20%) and aminopenicillins (12%) being prescribed most frequently[63]. The most common conditions for which antimicrobials were given included respiratory conditions, skin/mucosal conditions and UTIs[63]. Cystitis is a common presenting complaint in human medicine, effecting approximately 1 in 3 women by early adulthood and necessitating the use of antimicrobials[55]. Similar to the case in dogs, *E. coli* is the most common pathogen in uncomplicated cystitis cases[55].

1.2.4.1 Treatment guidelines from the human literature

In 2010, the Infectious Diseases Society of America (IDSA) updated their clinical guidelines for UTI treatment[64]. In general, there are significantly more options approved for the treatment of sporadic cystitis in human medicine. First line empirical treatments include the older agents fosfomycin, and nitrofurantoin as well as SXT and pivmecillinam. Second line treatment options include ciprofloxacin[64]. In contrast to the case in veterinary medicine, amoxicillin is no longer recommended, owing to its relatively poor efficacy, coupled with the high frequency (>20%) of resistance identified in previous guidelines[65,66]. Similar to veterinary guidelines, the IDSA no longer recommends fluoroquinolones for empirical treatment of UTIs citing its relative importance for infections other than cystitis[64]. A summary of IDSA treatment guidelines is given in Table 1.3.

Table 1.3: IDSA guidelines for the treatment of sporadic bacterial cystitis in women[64]

Drug	Class	Dose	Duration	Comments
Empirical treatment				
Nitrofurantoin	Nitrofuran	100 mg PO q12 for 5 days	5 days	Good first line choice. Comparable efficacy to trimethoprim-sulfamethoxazole
Fosfomycin	Phosphonic acid derivative	160/800 mg PO q12	3 days	Good first line choice. May have inferior efficacy compared with standard short- course regimens according to FDA
Trimethoprim-sulfamethoxazole	Potentiated sulfonamide	3 g PO once	-	Good first line choice. Use only in areas where local resistance rates are <20%
Pivmecillinam	β-lactam- extended spectrum penicillin	400 mg PO q12	3-7 days	Limited availability (not licensed in North America)
Second line treatments				
Ciprofloxacin/ofloxacin/ levofloxacin	Fluoroquinolone	-	3 days	Not recommended for routine use. Generally reserved for important uses other than cystitis
Amoxicillin-clavulanate, cefdinir, cefaclor, and cefpodoxime-proxetil	β-lactams	-	3-7 days	Generally inferior efficacy compared to other agents. Only recommended when agents cannot be used
Not Recommended				
Amoxicillin	β-lactam-aminopenicillin	-	-	Not recommended for routine use for uncomplicated cystitis due to widespread resistance and low efficacy

1.2.4.2 Alternative antimicrobials for the treatment of sporadic cystitis

Given the frequency with which antimicrobials are used to treat cystitis, surveillance initiatives for AMR in urinary pathogens have become common place in human medicine. Programs such as the North America Urinary Tract Infection Collaborative Alliance (NAUTICA) and the Study for Monitoring Antimicrobial Resistance Trends (SMART) have identified the rapid emergence and spread of resistance among community acquired urinary *E. coli* in Canada and the U.S., which has necessitated the investigation of alternative treatment options including the re-evaluation of older agents[67–72]. In fact, susceptibility testing in a 2013 U.S. study of 120 community acquired clinical *E. coli* isolates against six oral antimicrobials (azithromycin, doxycycline, minocycline, chloramphenicol, fosfomycin, and rifampin) considered older or alternative by current standards, revealed that resistance to fosfomycin was astonishingly rare, with in vitro susceptibility rates between 98-99%[73].

Fosfomycin is a bactericidal phosphonic acid derivative that was originally discovered in Spain in 1969[70,74]. Sold under the trade name Monurol, fosfomycin is approved exclusively for the treatment of uncomplicated cystitis in the U.S. and Canada[75]. However, there is a general lack of availability in parts of North America and many countries in Europe[76]. Fosfomycin is indicated for the treatment of infections caused by susceptible strains of *E. coli* and *E. faecalis* and is administered as a single oral dose containing 3 g of fosfomycin tromethamine[75]. This dosing regime is attractive clinically from the context of patient convenience and compliance, while simultaneously minimizing selective pressure for resistance as uropathogens are exposed for a relatively short period. This fact coupled with fosfomycin's unique mechanism of action

are reflected in the astonishing rates of susceptibility among community acquired uropathogens[77]. Fosfomycin has a broad spectrum of activity against both Gram-negative (Enterobacteriaceae) and Gram-positive (*Staphylococcus* spp. and *Enterococcus* spp.) pathogens that has been demonstrated in both in vitro and in vivo studies[69,71].

A 2010 study reviewed the microbiological and clinical literature regarding the efficacy of fosfomycin against Enterobacteriaceae isolates, including those with MDR phenotypes producing extended spectrum β-lactamase (ESBL) enzymes[78]. Of 13 microbiological studies, ten looked exclusively at community or hospital acquired UTIs where the vast majority of isolates were ESBL producing *E. coli*. All ten studies reported susceptibility rates in excess of 90%[79–88]. The authors concluded that among the species studied, *E. coli* was the most susceptible (96.8% of ESBL producing isolates). What's more, rates of resistance were higher overall among hospital acquired compared to community acquired strains[78]. Of the four clinical studies reviewed, two involved treatment of ESBL producing community and/or hospital acquired uropathogenic *E. coli*[87,89]. One study assessing fosfomycin in a three-dose protocol for the treatment of uncomplicated cystitis in patients who cultured positive for ESBL producing *E. coli*, reported clinical and microbiological cure rates of 94% and 78%[87]. Overall the cumulative cure rate (indicated by negative culture) in these studies was 93.8% causing the authors to conclude that fosfomycin is a valid and potentially useful treatment option for uncomplicated cystitis caused by ESBL producing *E. coli*[78]. The efficacy of fosfomycin was further assessed for the prevention of UTI recurrence in 166 patients compared to a placebo group. Patients in the treatment group received 3 g of fosfomycin

tromethamine every 10 days for 6 months. At one year follow up, the fosfomycin treated group reported a reinfection rate of 0.14 infections per year compared to 2.97 infections per year in the placebo group[90]. Additional recent studies have confirmed the efficacy of two and three dose fosfomycin protocols in both uncomplicated and/or complicated cystitis[91–93]. A single Canadian study assessing the in vitro activity of fosfomycin against a nationally representative sample of urinary *E. coli* concluded that 99.4% of isolates were susceptible to fosfomycin[94]. Susceptibility in MDR strains ranged from 95-100% among ESBL producers and 97-100% among AmpC β-lactamase producers[94]. Overall Fosfomycin represents a promising and efficacious option for the empirical treatment of UTIs caused by *E. coli*.

Despite its enthusiastic use in human medicine, literature documenting the efficacy of fosfomycin in treating canine infections is scarce. It is well known that fosfomycin tromethamine is predominantly excreted unchanged in the urine of humans, lending to its value in treating UTIs[70,74]. A single veterinary study investigating the pharmacokinetic properties of fosfomycin disodium in dogs failed to investigate urine concentrations, focusing instead on serum concentration and serum values that reflect kidney function[95]. What's more, the investigated agent is a parenteral formula when in reality the oral compound, fosfomycin tromethamine is used to treat cystitis. Canine subjects were administered fosfomycin at two different doses intravenously, intramuscularly, subcutaneously and orally once. The authors concluded that the only useful route, which achieved adequate plasma concentrations of fosfomycin was subcutaneous injection[95]. Given that fosfomycin is not currently approved for use in veterinary medicine, clinical trials have not been done in canine patients.

In vitro studies demonstrating the microbiological efficacy of fosfomycin against Enterobacteriaceae are scarce in veterinary medicine. A single study investigated the susceptibility of 200 clinical *E. coli* isolates from canine and feline infections; including UTIs, found astonishing rates of susceptibility, with just 3/200 isolates reported as resistant[96]. Experimental isolates (n=75) collected in the same study from the feces of dogs treated with either enrofloxacin or amoxicillin for 7 days exhibited 100% susceptibility to fosfomycin[96]. One other in vitro study investigating the susceptibility of *E. coli* isolated from bitches with pyometra found that 100% of isolates were susceptible[97]. The remainder of the veterinary literature investigating fosfomycin focuses on its efficacy against methicillin resistant *Staphylococcus pseudintermedius* (MRSP) in canine pyoderma cases. Susceptibility rates reported in these studies ranged from 77-100% among clinical isolates[98,99]. Interestingly, there has been a single case report of renal insufficiency in a cat following fosfomycin administration[100].

1.3 Antimicrobial resistance in veterinary medicine

Antibiotics are compounds produced by living organisms that inhibit microbial growth. The broader term antimicrobial, includes both synthetic and naturally derived compounds that exhibit activity against microorganisms, including antibacterials, antivirals, anthelminthics and antifungals[49]. The following section outlines the mechanisms of action of clinically important antimicrobials and provides a discussion of the most common mechanisms of resistance encountered against those agents used most commonly to treat canine cystitis.

1.3.1 Mechanisms of antimicrobial action

Antimicrobials can be grouped broadly based on their primary site of action in the bacterial cell. Antimicrobial mechanisms of action include protein synthesis inhibition, cell wall synthesis inhibition, inhibitors of DNA metabolism and disruption of cell membrane structure[49]. Additional compounds have also been developed to combat highly prevalent AMR mechanisms including the group of compounds known to inhibit β-lactamase enzymes (clavulanic acid, sulbactam, tazobactam)[101]. Antimicrobials vary in their spectrum of activity. While some exhibit specific activity against Gram-negative or Gram-positive microorganisms, others have a broad spectrum of activity against many different microbial species[49].

1.3.1.1 Protein synthesis inhibitors

Protein synthesis inhibitors act on the cellular machinery involved in translating mRNA into polypeptide chains (proteins). The primary site of action of these drugs is the ribosome[101]. In contrast to the larger 80S ribosome in eukaryotes, bacteria have a 70S ribosome that consists of a 50S subunit and a smaller 30S subunit[101]. Either of these may be the primary site of action of antimicrobials. Examples of protein synthesis inhibitors approved for use in veterinary species include the aminoglycosides (gentamicin and amikacin), macrolides (erythromycin, clarithromycin and clindamycin), tetracyclines (oxytetracycline), phenicols (chloramphenicol), streptogramins and lincosamides[101].

1.3.1.2 Cell wall synthesis inhibitors

Cell wall synthesis inhibitors target the enzymes and substrates involved in peptidoglycan synthesis, a structure that is absent in eukaryotes and specific to bacteria[101]. Important classes, which act by this mechanism include the very large β-lactam family, fosfomycin and glycopeptides (vancomycin)[101]. β-lactams can be further broken down into penicillins (amoxicillin, ampicillin), cephalosporins (cephalexin, ceftiofur, ceftriaxone), cephamycins (cefoxitin), monobactams (aztreonam) and carbapenems (meropenem)[102]. These compounds act similarly owing to a common four membered ring in their structure, by binding penicillin binding proteins involved in cell wall synthesis[101,102]. In fact, this common structure has implications for the acquisition of resistance to this class that has resulted in the development of many iterations of cephalosporins that can be grouped into different generations based on their structure[101]. For example, the extended spectrum cephalosporins (ESC), initially developed in the 1980s, include a bulky oxyimino subgroup in their structure, which, at least temporarily evaded the development of resistance to these compounds[102]. Carbapenems are a critically important antimicrobial class for the last resort treatment of human infections and so are not widely used in veterinary medicine[103].

In addition to the cell wall, bacteria possess a cell membrane which can be the target of antimicrobial compounds. The polymyxins (ex. Colistin) are one such example of a drug class, which exert their mechanism of action by interfering with cell membrane structure[101].

1.3.1.3 Inhibition of DNA metabolism

DNA metabolism encompasses both DNA replication and folic acid metabolism. The fluoroquinolones and rifampin are examples of compounds which interfere with enzymes involved in DNA replication[101,103]. While rifampin acts on the DNA polymerase that synthesizes new DNA strands, fluoroquinolones act on DNA gyrases involved in relaxing the supercoiled structure of DNA prior to replication[101,103]. Antimicrobials that competitively inhibit the synthesis of folic acid include the sulfonamides (sulfamethoxazole) and trimethoprim[101].

1.3.1.4 β-lactamase inhibitors

Fully synthetic antimicrobial compounds have also been developed to specifically target certain enzymes produced by AMR bacteria. These β-lactamase inhibitors (ex. clavulanic acid, tazobactam, sulbactam and boric acid) are always administered in combination with β-lactam antimicrobials and exert their action by irreversibly binding β-lactamase enzymes capable of breaking down the β-lactam antimicrobial, thus making these drugs more effective[101].

1.3.2 Mechanisms of antimicrobial resistance

Antimicrobial resistance is a natural phenomenon that predates the clinical use of antimicrobial compounds. Certain microbes are intrinsically resistant to the action of antimicrobial drugs due to their inherent structure or physiology. For example, *Mycoplasma spp.* are resistant to the action of cell wall synthesis inhibitors (ex. β-lactams) as they lack a cell wall[49]. On the other hand, AMR can also be acquired.

Microorganisms may develop resistance by spontaneous mutations in their chromosomal DNA, for example through the occurrence of a single nucleotide polymorphisms (SNP). However, bacteria also have the ability to acquire resistance genes horizontally form other microorganisms[49]. Horizontal gene transfer may occur via transformation, transduction or conjugation on plasmids or transposons. Plasmids are a form of extrachromosomal DNA that replicate apart from the chromosome and that are freely exchanged between and within bacterial species[49]. Transposons, are pieces of chromosomal DNA, which are capable of excising themselves from the DNA of a donor organism and inserting into either the plasmid or chromosomal DNA of a recipient[52]. Transformation refers to the uptake of naked DNA from the environment, while transduction involves the acquisition of resistance genes from viruses. Finally, conjugation involves the transfer of mobile genetic elements between two bacteria in direct contact[101].

Mechanisms of AMR fall into five major categories; i) changes in cell membrane permeability or transporters decreasing antimicrobial uptake ii) expression of efflux pumps increasing the removal of antimicrobials from the cell iii) the acquisition of enzymes that are capable of hydrolyzing or otherwise inactivating antimicrobials iv) alterations of the antimicrobial target by mutation or target protection v) immunity and bypass of antimicrobial sites of action caused by the over expression of antimicrobial targets or the development of alternative metabolic pathways[101,103]. The following sections discuss each of these mechanisms in detail, using *E. coli* as an example.

1.3.2.1 Decreased uptake

Intrinsic differences in permeability exist between bacterial species. For example, the outer membrane of Gram-negative bacteria (which is absent in Gram-positive bacteria) provides an additional permeability barrier to the uptake of antimicrobial agents. These organisms represent a particular challenge in developing new treatment options[101]. Decreased uptake of the cell wall synthesis inhibitor fosfomycin is a common mechanism of resistance in *E. coli*. This agent is reliant on two different transport systems (a hexose phosphate transporter and a glycerol-3-phosphate transporter) for uptake into the cell[76]. Acquired mutations in the genes encoding one or both of these transporters results in resistance[103]. What's more, outer membrane protein (OMP) deficient strains of Enterobacteriaceae, including *E. coli*, have also emerged recently, which confer resistance to the critically important carbapenem β-lactams[104,105].

1.3.2.2 Efflux pumps

Various efflux pumps have been described, which are capable of transporting different antimicrobial classes. While some, such as the fluoroquinolone efflux pump encoded by *qep*A, are highly specific, others are capable of transporting a wide variety of compounds. For example, the *oqx*AB gene encodes a transmembrane protein that is capable of transporting quinolones, tetracyclines chloramphenicol and trimethoprim[103,106]. Genes encoding these transporters are often acquired by horizontal gene transfer. For example, in *E. coli,* tetracycline drugs may be removed from the cell by acquisition of a variety of *tet* genes. To date, nine genes encoding tetracycline transporters have been identified (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*J, *tet*L and *tet*Y)[103].

Examples of efflux pumps against other drug classes include the *flo*R gene which encodes a phenicol transporters[103].

1.3.2.3 Enzymatic inactivation

Currently, AMR mediated by enzymatic inactivation is one of the most important mechanisms threatening the clinical utility of antimicrobials. These enzymes exist in both Gram-negative and Gram-positive organisms[52,107]. For example, penicillinase enzymes acquired by certain strains of *Staphylococci* confer resistance to penicillin antimicrobials[52]. However, particularly among Gram-negative pathogens, inactivating enzymes are both prevalent and permissive. Enzymes have been identified encoded on transferrable genetic elements, which confer resistance to β-lactams (β-lactamases), aminoglycosides (aminoglycoside aminotransferases), fosfomycin (*fos*A, *fos*B, *fos*X), tetracyclines (*tet*X), chloramphenicol (*cat* genes) and fluoroquinolones (*aac(6')-Ib-cr*)[103].

1.3.2.4 Target alterations

Antimicrobial sites of action can be altered in two distinct ways. Chromosomal mutations in the genes encoding the target can lead to decreased affinity or activity of an antimicrobial. For example, Gram-negative organisms develop decreased susceptibility to fluoroquinolones by acquiring mutations in the *par*C and *gyr*A genes which encode domains of the DNA gyrase enzymes upon which fluoroquinolones act[103]. Mutations in the *rmt* and *arm*A genes have also been described, which confer resistance to aminoglycosides[103]. It is also possible for an antimicrobial target to become modified by some acquired mechanism (a protein or enzyme) that protects it from the activity of the antimicrobial. Examples where resistance is conferred in this manner have been

described in *E. coli* for tetracyclines (*tet*M and *tet*W), fluoroquinolones (*qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S), phenicols, macrolides and oxazolidinones (*cfr*)[103].

1.3.2.5 Immunity and bypass

The sulfonamides and trimethoprim are the most important example of an antimicrobial class that exert their action by interfering with cellular metabolism, specifically folic acid synthesis[103]. Resistance to either of these compounds in isolation can occur in Enterobacteriaceae through the acquisition or enzymes which provide an alternative pathway to complete folic acid synthesis. For example, while the sulfonamides are active against dihydrofolate reductase (encoded by *dfr* genes), trimethoprim is active against dihydropteroate synthetase (encoded by *sul* genes). Therefore, the acquisition of *sul* genes confers resistance to sulfonamides while the acquisition of *dfr* genes confer resistance to trimethoprim[103]. In order to more effectively target folic acid synthesis, “potentiated” sulfonamide formulations were developed which combine these two agents, for example, trimethoprim-sulfamethoxazole.

1.3.3 Antimicrobial resistance in urinary *E. coli*: A clinical perspective

The mechanisms responsible for AMR are diverse across microbial species. However, antimicrobial exposure in any context provides a common selective pressure for the emergence and dissemination of resistance in bacterial population. From a clinical perspective, certain pathogens and antimicrobials are more important than others[3]. According to the Center for Disease Control and Prevention (CDC) ESBL producing Enterobacteriaceae pose a serious threat to public health in the context of AMR[3]. Within the family Enterobacteriaceae, the versatile organism *E. coli*, is of interest

for a variety of reasons. First, despite being intrinsically susceptible to many clinically relevant antimicrobials, *E. coli* has an exceptionally plastic genome and is able to both acquire and spread resistance genes by horizontal gene transfer. *E. coli*, therefore represents an important reservoir of resistance genes within microbial communities[103]. Second, the transmission of *E. coli* between and within different hosts can occur by a variety of mechanisms including direct contact or via contaminated food and water sources[103]. Third, *E. coli* represent both a commensal organism and pathogen. The pathogenic potential of *E. coli* varies significantly and can be classified into different pathotypes based on the anatomic site of disease[103]. While the majority of *E. coli* that is ubiquitous in the gastrointestinal tract of humans and animals are benign, some strains are capable of causing disease (diarrhea and colitis) and belong to pathotypes known as enterotoxigenic *E. coli* (ETEC). This is in contrast to extra-intestinal pathogenic *E. coli* (ExPEC), which migrate from the digestive tract to extra-intestinal sites, such as the urogenital tract and blood stream, to cause disease[103]. The *E. coli* genome normally comprises between 4,000 and 5,000 genes, of which approximately 3,000 are conserved across strains. The remainder are responsible for the spectrum of pathogenicity observed among *E. coli* isolates and comprise virulence and colonization determinants that improve the disease-causing potential of their host[103].

As a ubiquitous organism with genetic plasticity and significant pathogenic potential, there are many resistance mechanisms worth investigating in *E. coli*. The most relevant currently are ESBLs, carbapenemases, and plasmid-mediated quinolone resistance (PMQR) genes, which are present on mobile genetic elements that are exchanged between and within bacterial species[103]. Additional transmissible resistance

mechanisms are described with increasing frequency against fosfomycin, which has re-emerged in human medicine for the treatment of sporadic cystitis[103]. This section describes drug specific resistance mechanisms in *E. coli* in more detail from the perspective of veterinary medicine.

1.3.3.1 β-lactams

The β-lactams are a large drug class that includes penicillins, cephalosporins, monobactams and carbapenems[102].They are named for the common presence of a four membered β-lactam ring[102]. Enzymatic inactivation by β-lactamases are the most important mechanism of resistance to this drug class, particularly among Enterobacteriaceae[103]. These enzymes are of particular concern for a number of reasons. First, they target a common structural component in β-lactam drugs, the β-lactam ring and thus, they display activity against a wide variety of these compounds[108]. Second, β-lactamases are both chromosomally and plasmid encoded and therefore have the potential to be freely exchanged between and within bacterial species[109–111]. Third, there is great diversity among β-lactamases in terms of their spectrum of activity against β-lactam drugs[109]. These enzymes are classified most commonly using the Ambler system, which takes into consideration their molecular structure and amino acid sequence[112]. However, functional classifications based on hydrolytic spectra also exist[113]. Ambler class A, C and D enzymes, though significantly different in their amino acid sequence, contain a common serine residue in their active site. Class B enzymes, on the other hand, utilize a divalent zinc ions for substrate hydrolysis[113,114]. Class A includes the narrow spectrum penicillinase type β-lactamases (TEM-1), ESBLs and KPC

type enzymes. Class B contains the metallo-β-lactamases, while Class C includes plasmid mediated AmpC β-lactamases. Finally, Class D includes the oxacillinases, also known as OXA-48 like enzymes[110,115,116]. Based on spectrum of activity these enzymes are more accurately divided into ESBLs, carbapenemases (including, KPC, metallo β-lactamases, and OXA-48 like enzyme) and plasmid mediated AmpC β-lactamases[110]. The major classes of β-lactamases described in *E. coli* are outlined in Table 1.4[110,115].

Table 1.4: An overview of β-lactamase enzymes in *E. coli*[110,115]

Enzyme	Classification	Example	Phenotype	Inhibition
Extended spectrum β-lactamases (ESBLs)	Class A	CTX-M, SHV, TEM	Penicillins Cephalosporins Monobactams	Clavulanic acid Tazobactam Sulbactam
AmpC β-lactamases	Class C	CMY, FOX, ACT, MOX, ACC, DHA	Penicillins Cephalosporins Monobactams Cephamycins	Cloxacillin Boronic acid
Carbapenemases				
Metallo-β-lactamases (MBLs)	Class B	IMP, VIM, NDM	Penicillins Cephalosporins Cephamycins Carbapenems	Metal chelators (EDTA)
KPC carbapenemases	Class A	KPC	Penicillins Cephalosporins Cephamycins Carbapenems	Clavulanic acid (weak) Tazobactam Boronic acid
OXA-β-lactamases	Class D	OXA-48, OXA-181	Penicillins Carbapenems	Clavulanic acid (weak) NaCl

1.3.3.1.1 Extended spectrum β-lactamases

ESBLs are capable of hydrolyzing even those extended-spectrum β-lactams that had been modified to confer resistance to the first generation of β-lactamase enzymes[110]. They exhibit activity against penicillins, monobactams and cephalosporins, including ESCs, but remain susceptible to cephamycins, carbapenems and β-lactamase inhibitors such as clavulanic acid[110]. SHV, TEM and CTX-M type ESBLs are among the most common. While SHV and TEM type enzymes dominated originally, since the beginning of the 21st century, CTX-M type enzymes have become most prevalent[76,110,117].

1.3.3.1.2 AmpC β-lactamases

Class C β-lactamases (or AmpC β-lactamases) are widespread in bacterial species and may be both chromosomally and plasmid encoded[116]. Specifically, *E. coli* tend to possess chromosomally encoded, non-inducible AmpC β-lactamases which may be upregulated by mutations in the promoter[76,110,118]. *E. coli* may also acquire plasmid mediated AmpC β-lactamases of which the CMY-2 type is most common[116,119]. AmpC β-lactamases confer resistance to penicllins, cephalosporins, monobactams and cephamycins, as well as the “classic” β-lactamase inhibitors such as clavulanic acid. However, they remain susceptible to fourth generation cephalosporins and carbapenems[76]. Examples of AmpC β-lactamases include CMY, ACT, FOX, MOX, ACC and DHA type enzymes[76,110]. CMY-2 is the most common type detected in companion animals, having been identified in dogs from Japan, Denmark and Canada[120–122].

1.3.3.1.3 Carbapenemases

The carbapenemase designation most accurately describes a phenotype, which includes resistance to the carbapenem antimicrobials (meropenem and imipenem). These enzymes are phylogenetically diverse and belong to three different Ambler classes including KPC-type (Class A) metallo β-lactamase type (Class B) and OXA-48 type (Class D) enzymes[76,110]. KPC type carbapenemases have a broad spectrum of activity. They hydrolyze penicillins cephalosporins, cephamycins and carbapenems but are inhibited by tazobactam and boronic acid[76,110]. To date a single KPC producing *E. coli* isolate has been identified in a companion animal, originating from a dog suffering from recurrent UTIs in Brazil[123]. Metallo β-lactamases are capable of breaking down penicillins cephalosporins, cephamycins and carbapenems. However, due to their structure, they are susceptible to inhibition by metal chelators such as EDTA. Examples of metallo β-lactamases include NDM, VIM and IMP type enzymes[76,110]. To date NDM-1 and NDM-5 type enzymes have been identified in companion animals. NDM-5 was isolated from the feces of a healthy dog in Algeria, while NDM-1 was identified in clinical isolates from dogs in China and the U.S.[124–126]. OXA-48 type enzymes have a complicated phenotype exhibiting activity against penicillins, carbapenems and β-lactamase inhibitors but only weakly inhibiting monobactams and ESCs. OXA-48 type carbapenemases are inhibited by NaCl[110]. OXA-48 has been identified in *E. coli* of companion animal origin in France, Germany, Algeria and the U.S.[127–130].

1.3.3.2 Fluoroquinolones

The fluoroquinolones are a broad-spectrum antimicrobial class that exert their mechanism of action by interfering with DNA replication[49]. Originally, acquired resistance to fluoroquinolones was thought to occur strictly through chromosomal mutation in the genes *gyr*A and *par*C that encode the drug target[45,131]. The first report of PMQR originated on an MDR plasmid (pMG252) found incidently in a ciprofloxacin resistant urinary isolate of *K. pneumoniae* in Alabama in 1994[132]. The protein encoded by this gene, (later designated *qnr*A1), confered reduced susceptibility rather than full clinical resistance on its own. However, wild type *E. coli* to whom pMG250 was transferred were 100 times more likely to develop spontaneous mutations when exposed in vitro to ciprofloxicin or nalidixic acid[132].

To date, three types of transmissible quinolone resistance genes have been reported. The target protecting protein (*qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S); the inactivating enzyme ***aac(6')- Ib-cr***, and the efflux pumps *qep*A and *oqx*AB[45]. All of these PMQR determinants technically confer reduced susceptibility, indicated by an increase in MIC. However, subsequent quinolone (or fluoroquinolone) exposure exerts a selective pressure for the acquisition of additional mutations that often result in clinical resistance[133]. The efflux pump *oqx*AB is an interesting case as it actually has an affinity for multiple compounds in addition to (fluoro)quinolones[103]. This determinant therefore provides a mechanism by which reduced susceptibility to multiple classes of antimicrobials, including trimethoprim and chloramphenicol, may be co-selected[103]. A variant of an aminoglycoside acetyltransferase enzyme, AAC(6')IB-CR, that is capable of hydrolyzing ciprofloxacin, was first described in 2005[134]. Currently, the ***aac(6')- Ib-cr***

gene appears to be the most widespread in companion animals, having been identified in *E. coli* from both healthy and diseased animals across Europe, Australia, Asia and the U.S.[135–139]. The tendency of the *aac(6')- Ib-cr* gene to co-locate on plasmids harbouring ESBLs is of particular concern as it provides the framework for the emergence of MDR. These strains have been commonly identified in dogs with UTIs in Asia and Europe[135–138]. Many additional PMQR determinants have been detected in dogs in China, Europe, Australia and the U.S.[136,137,139–143].

1.3.3.3 Fosfomycin

Fosfomycin acts by inhibiting UDP N acetyl glucosamine enolpyruvyl transferase (MurA), an enzyme required for the first step of peptidoglycan biosynthesis[76]. In *E. coli,* recognized fosfomycin resistance mechanisms include decreased uptake and enzymatic inactivation[76,103]. Target modification by chromosomal mutations in the *mur*A gene has also been described[76,103]. Specifically, mutations in the hexose phosphate (*uhp*A and *uhp*T genes) and glycerol 3 phosphate (*glp*T gene) transporter system involved in fosfomycin uptake lead to decreased susceptibility or clinical resistance. Multiple fosfomycin-modifying enzymes; including a glutathione transferase (FosA), a thiol transferase (FosB) a hydrolase (FosX) and two kinases (FomA and FomB) have also been described in *E. coli* [76,103]. Many of the genes encoding these enzymes have been identified on plasmids in Gram-negative (*fos*A) and Gram-positive (*fos*B) organisms, alongside additional resistance determinants[144,145]. Therefore, fosfomycin resistance can be co-selected for in the face of exposure to other antimicrobial agents[103]. Investigations into fosfomycin resistance in companion animals are relatively recent. After the initial discover of *fos*A in dogs and cats in China in 2012 and 2013, *fos*A has emerged as the

most commonly identified fosfomycin resistance mechanism among companion animals and appears to be particularly widespread among canine populations in China[146–148].

1.4 Antimicrobial resistance surveillance in human urinary tract infections

The CDC refers to surveillance as 'the ongoing systematic collection, analysis, and interpretation of data essential to the planning, implementation, and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know'[149]. In Canada, national AMR surveillance is under multiple initiatives including the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), and the Canadian Antimicrobial Resistance Surveillance System (CARSS). What's more, coordinated multicentre initiatives for tracking AMR in both health care associated, and community acquired UTIs are well established in human medicine. The North America Urinary Tract Infection Collaborative Alliance (NAUTICA), the Canadian Ward Surveillance Study (CANWARD), the Study for Monitoring Antimicrobial Resistance Trends (SMART) and the Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) program are several of the prominent programs active in North America.

1.4.1 North America

In Canada, the CANWARD study (initiated in 2007) collects isolates from ten participating Canadian hospitals across eight provinces. The most recent resistance trends among in and outpatient urinary pathogens collected through the program from

2007-2009 indicate that *E. coli* accounted for 43% and 64% of positive cultures in inpatient and outpatient UTI cases respectively[150]. Overall, among all uropathogens, susceptibility rates to nitrofurantoin (96%) and ciprofloxacin (81%) remained high. Over the three-year study period for *E. coli* specifically, statistically significant increases in resistance occurred only for amoxicillin-clavulanate and SXT. In addition, 12.2% of *E. coli* were found to be MDR with ciprofloxacin and SXT resistance being over represented (95% and 92% respectively) among co-resistant isolates[150]. In comparison to earlier Canadian studies, the prevalence of *E. coli* resistant to ciprofloxacin has increased by more than sixteen times, while nitrofurantoin resistance has increased more than twenty fold[68,150,151]. In contrast, resistance to SXT has remained relatively stable over the past decade[68,150,151]. A summary of the changes in resistance to nitrofurantoin, ciprofloxacin and SXT in Canadian urinary *E. coli* from 1998-2011 is summarized in Table 1.5[68,150,151]. SMART reported a similar rate of fluoroquinolone susceptibility (71%) among urinary pathogens collected in Canada and the U.S. between 2010 and 2014. Suggesting that this trend of decreasing efficacy of fluoroquinolones for the treatment of UTIs has continued into the present decade[152]. Additional studies conducted over a ten year period in Canada and the U.S. report similar changes in fluoroquinolone resistance from 1.2% to 19.2% and 3% to 17% in Canada and the U.S. respectively between 2000 and 2010[150,151,153].

Table 1.5: Changes in the frequency of nitrofurantoin, ciprofloxacin and SXT resistance among urinary *E. coli* in Canada[68,150,151]

	1998	2003-2004	2007-2009
Nitrofurantoin	0.1%	0%	4.1%
Ciprofloxacin	1.2%	1.1%	19.5%
Trimethoprim-sulfamethoxazole	18.9%	17.7%	22.1%
Prevalence of E. coli	84.1%	56.9%	54%
Total isolates (N)	1681	496	1581

In Canada, antimicrobial susceptibility of community acquired urinary *E. coli* has been shown to vary significantly in comparison, to the United States. For example, American isolates exhibit significantly higher rates of resistance to ampicillin (39.3% versus 33.0%), SXT (22.6% versus 17.7%) nitrofurantoin (1.4% versus 0%), and ciprofloxacin (6.8% versus 1.1%)[154]. This finding was supported by the more recent SMART study (2010-2014) which found that among *E. coli* collected from 2013-2014, resistance to cephalosporins and fluoroquinolones was significantly higher among American isolates by about 4% and 6% respectively[152]. There was also significant geographical differences regarding risk of quinolone resistance between regions in the U.S. with an approximately 50% increased prevalence among isolates originating from the south[155]. Interestingly, similar variation has also been documented across Canada, with the highest rates of multidrug, as well as fluoroquinolone resistance occurring among urinary *E. coli* in British Columbia[156,157].

1.4.2 Global

The Antimicrobial Resistance Epidemiological Survey on Cystitis (ARESC) is an international survey to investigate the prevalence and epidemiology of uropathogens causing cystitis worldwide[158]. Among isolates collected from women with uncomplicated cystitis in nine European countries as well as Brazil (2003-2006), *E. coli* accounted for 76.7% of isolates. Overall, resistance to SXT (29.4%) and ampicillin (48.3%) were most common. Fosfomycin, pivmecillinam and nitrofurantoin were the most efficacious with susceptibility rates above 90%[158]. Among participating countries, resistance to ciprofloxacin exceeded 10% in Brazil, Spain Italy and Russia[158]. In general, the greatest geographic variability in susceptibility was observed for SXT (54.5-87.8%) and members

of the β-lactam family including ampicillin (32.7–65.5%), amoxicillin-clavulanate (51.9–93.5%), and cefuroxime (73.0–93.0%)[158].

The European *E. coli* Sensitivity survey (ECO.SENS) is a longitudinal surveillance project focused specifically on tracking the susceptibility of *E. coli* from female patients with uncomplicated cystitis in five European nations[159]. Most recently, results from 2014 were compared to susceptibility rates in 2000 and 2008 respectively. Since 2000, there has been a significant increase in resistance to the first-generation cephalosporin cefadroxil in Germany and Spain, to ciprofloxacin in Germany, Spain, Sweden, and the UK, to SXT in Germany, Spain, Sweden, and the UK, to pivmecillinam in Spain, and to nitrofurantoin in the UK[159]. Since 2008, the most astonishing increases in resistance have occurred in the UK for ciprofloxacin, SXT and nitrofurantoin[159].

1.4.3 Antimicrobial resistance and antimicrobial use

While the situation in any particular location may vary, some consistent AMR trends have been identified among urinary *E. coli* globally. i) Resistance rates are higher among hospital acquired compared to community acquired infections; ii) Resistance to oral formulations of fluoroquinolones, β-lactams (including aminopenicillins, potentiated aminopenicillins and first generation cephalosporins) and SXT commonly used to treat UTIs is increasing; iii) Susceptibility to fosfomycin, pivmecillinam and nitrofurantoin remain high (>90%) in most regions; and iv) The frequency of MDR is increasing and non-susceptibility to fluoroquinolones is over represented among these isolates.

Many of these observations appear to have been taken into consideration in the most recent modifications to the international UTI treatment guidelines published by the IDSA[64]. In summary, amino penicillins (amoxicillin) and their potentiated counterparts (amoxicillin-clavulanate) are no longer recommended for empirical treatment. Furthermore, fluoroquinolones are now considered a second line treatment option owing to the increasing reports of resistance and their relative importance in treating infections other than cystitis[64]. Current empirical treatment options instead include fosfomycin, nitrofurantoin, pivmecillinam and SXT (where regional resistance rates are <20%)[64]. Despite the “international” nature of these guidelines, significant differences in regional prescribing practices still exist[30]. For example, pivmecillinam is largely unavailable in North America, but is commonly prescribed in Europe[64].

National treatment guidelines for uncomplicated cystitis in six European countries recommend seven different antimicrobials. However, no single drug is consistently recommended across all six nations suggesting that regional differences in the frequency of resistance affect how guidelines are developed and applied[160]. For example, in Spain, which is a country known for having a significant AMR problem, ciprofloxacin resistance rates among urinary *E. coli* exceeded 20%, yet national guidelines continue to recommend fluoroquinolones as first line empirical treatments for uncomplicated infections[160,161]. Interestingly despite its widespread availability and more extensive label claims in Europe, the only two European nations currently recommending fosfomycin for first line treatment are Spain and Germany[160].

1.4.4 Molecular epidemiology of resistance in urinary *E. coli*

AMU provides the selective pressure for bacteria to develop resistance. The most common general mechanisms of AMR include decreased permeability, efflux pumps, target protection, target modification, enzymatic inactivation and immunity and bypass. Here, an overview of relevant molecular techniques for investigating AMR is provided and the epidemiology of resistance genes identified in human urinary *E. coli* is discussed. Special attention is paid to those mechanisms which confer resistance to the β-lactams and fluoroquinolones which are relevant for treating UTIs.

1.4.4.1 An overview of molecular microbiology techniques

The identification of phenotypic AMR, while often considered sufficient for clinical purposes, provides an incomplete description of how and why resistance occurs. Advances in molecular microbiological techniques in the past decade have allowed for more extensive investigations into the mechanism and epidemiology of resistance. Many of these techniques are based on polymerase chain reaction (PCR), sequencing, restriction digestion, or some combination. PCR is a relatively fast and cheap way to identify a gene of interest utilizing short DNA segments (primers) that bind to complementary sequences on either end of a specific target. A PCR reaction proceeds through many cycles to exponentially amplify the target. At the end, the reaction mixture is loaded onto an agarose gel and exposed to an electric field that causes the DNA to migrate down the gel. The amplified gene is detected as a band when the gel is imaged in the presence of ultraviolet light. PCR methodology can be further exploited to gain insight into the genetic relatedness of isolates. For example, multilocus sequence typing

(MLST) is a PCR based technique that detects sequence difference in conserved genes to determine epidemiological relationships between members of a bacterial species[162]. In short, for each isolate, PCR is carried out on seven conserved housekeeping genes and the purified products are sequenced. When the sequence results are compared to an MLST database (pubMLST), a distinct allelic profile is assigned to each isolate based on the combination of seven unique sequences. From this information each isolate can be assigned a sequence type (for example, ST131) which defines a cluster of closely related strains. Alternatively, pulse field gel electrophoresis (PFGE) may be used for DNA fingerprinting and is an extension of traditional agarose gel electrophoresis. Briefly, bacteria from overnight culture is subjected to cell lysis and suspended in plugs of agarose gel. Plugs are subjected to restriction digestion by commercially available enzymes and loaded into an agarose gel[163]. A constantly changing electric field is applied to separate the resulting large DNA fragments and the gel is stained and imaged in the presence of UV light[163]. The observed banding pattern can be compared to a database (PulseNet) of known PFGE profiles, which allows the relationship between clinical isolates and reference strains to be determined. These various DNA fingerprinting techniques have revolutionized microbiology and allowed for extensive digitization and sharing of information for epidemiological purposes.

1.4.4.2 β-lactamases

β-lactamase is a broad term which encompass all enzymes capable of breaking down the various antimicrobials belonging to the β-lactam class. With respect to uropathogenic *E. coli*, the most problematic β-lactamases are those with a broad spectrum of activity including ESBLs and AmpC β-lactamases[76]. Interestingly, it is

possible for *E. coli* to possess multiple β-lactamase genes which are only detected by molecular methods[164].

1.4.4.2.1 Extended spectrum β-lactamases

There are multiple types of ESBLs which including SHV, TEM, and CTX-M types. Of these classes, CTX-M type enzymes are particularly prevalent among both hospital and community acquired UTIs caused by *E. coli* [76].

In North America, the first report of a CTX-M producing urinary *E. coli* isolate occurred in 2002 when 9 clinical isolates (six of which were from the urinary tract) from five different states were found to possess these enzymes[165]. Since their initial discovery, screening for ESBLs has become a common part of UTI surveillance studies in human medicine. In Canada, rates of CTX-M producing urinary *E. coli* are relatively low. Among 208 *E. coli* isolates collected from women with uncomplicated cystitis in 167 towns across Canada, one (0.5%) phenotypic ESBL producer was identified[156]. Another Canadian study investigating ESBL production among *E. coli* from 11 medical centers across Canada identified 209 ESBLs of which 78% were recovered from urine and 62% were collected from community acquired infections[166]. Overall, CTX-M type enzymes comprised 82% of ESBLs, of which the majority (71%) were CTX-M-15[166]. Some of these CTX-M-15 producers carried additional β-lactamases including those of the TEM and OXA types. The remaining 38 isolates possessed various alleles of the SHV and TEM type ESBLs[166]. The SMART study surveying for ESBL production among hospital and community acquired urinary *E. coli* in both Canada and the U.S. identified a significant increase in the frequency of ESBLs in the U.S. from 7.8% to 18.3% between

2010 and 2014[152]. In Canada, this increase (10.4% to 13%) did not reach statistical significance. However, when just community acquired infections caused by ESBL producers was considered, the increase in frequency was significant in both countries[152]. The occurrence of ESBL production among community acquired uropathogens was approximately 15% in both countries as of 2015[152]. Upon further molecular characterization of ESBL producers, 90% possessed CTX-M enzymes of which the majority were CTX-M-15, followed by CTX-M 14 and CTX-M 27. SHV and TEM type enzymes accounted for a minority of isolates (~5%) in both the U.S. and Canada[152]. Interestingly, in this same study, two isolates producing KPC type carbapenemases were identified and both originated from the U.S[152].

For global comparison, rates of ESBL production among community acquired UTIs in Spain jumped from 7.5% in 2010 to 8.6% in 2012 and this increase was even more pronounced for hospital acquired UTIs[161]. Among urinary *E. coli* isolates collected from a tertiary care hospital in India, 23% were found to harbor ESBLs of which 16% produced CTX-M type enzymes[167]. The most recent results from the ECO.SENS initiative identified a phenotypic ESBL frequency of 4.8% among 729 isolates collected from five European countries between 2008-2014[159]. Interestingly, previous results from the ECO.SENS study, which characterized ESC resistance in isolates from both high AMR burden (Greece and Portugal) and low AMR burden (UK, Austria and Sweden) countries found that among 11 (0.6%) ESC resistant isolates, five produced CTX-M group 1 and one produced CTX-M group 9 type enzymes[168]. Interestingly, at this time there was at least one ESBL producing isolate identified from each country except Portugal[168].

1.4.4.2.2 AmpC β-lactamases

The prevalence of Amp-C β-lactamases is under investigated in Canada. To the authors knowledge no studies have specifically investigated the frequency of plasmid mediated AmpC β-lactamases among urinary *E. coli*. However, one study identified 232 cefoxitin (a cephamycin) resistant clinical *E. coli* isolates from Canadian hospitals, which accounted for just 0.8% of the total[169]. Molecular characterization revealed 25 (11%) strains that possessed CMY-2 type AmpC β-lactamases[169]. CMY-2 type plasmid mediated AmpC β-lactamases have also been reported in the UK, China and the United States[170–172].

1.4.4.3 Plasmid mediated quinolone resistance determinants

The frequency of fluoroquinolone resistance among Enterobacteriaceae has increased significantly in the last twenty years[45]. This observation, coupled with the significant association between fluoroquinolone resistance and MDR phenotypes has triggered broad scale investigations into the prevalence of the plasmid mediated quinolone resistance (PMQR) determinants *qnr*A, *qnr*B, *qnr*C, *qnr*D *qnr*S, ***aac(6')- Ib-cr***, and *qep*A[45,155].

Surveillance for PMQR in Canada is most extensive in the Calgary Health Region. An investigation into the occurrence of PMQR among fluoroquinolone resistant clinical *E. coli* isolated from 2004-2007, revealed that the frequency of the ***aac(6')-Ib-cr*** gene increased from 4% in 2004 to 13% in 2007[173]. In 2007, 0.8% of isolates were positive for the *qnr*S gene and one isolate was positive for both genes. All PMQR determinants were present in urines and the majority were submitted from community

acquired infections[173]. Interestingly, 13% of isolates co-produced AmpC β-lactamases and 54% co-produced CTX-M-15 type ESBLs[173]. In fact, cotransmission of quinolone resistance with aminoglycoside inactivating enzymes and ESBLs is quite well document and has the potential to facilitate the rapid emergence of MDR[174]. An additional study from the same region investigated the prevalence of PMQR among ESBL producing *E. coli*, 78% of which originated from urine[166]. Eighty nine percent of these ESBL producers exhibited resistance to ciprofloxacin among which 54% produced the *aac(6')- Ib-cr* gene and a single isolate produced both the *aac(6')- Ib-cr*, and *qnr*B genes[166]. The final PMQR gene *qep*A, which encodes an efflux pump, was first reported in North America in an *E. coli* isolate in Canada[175]. While investigating mechanisms of resistance among ESBL producing *E. coli* isolated in ICU's across Canada, PMQR genes *qnr* (A, B, and S), *qep*A, and *aac(6')-Ib-cr* were detected in 0%, 5.6%, and 44.4% of isolates respectively. Interestingly 100% of isolates also harbored chromosomal mutations in the quinolone resistance determining region of *gyr*A, while 88.9% of isolates had mutations in *par*C[175]. Unfortunately, the specimen types from which these MDR *E. coli* were isolated was not described.

Internationally, *aac(6')- Ib-cr* is the most widespread of the PMQR determinants[106]. However, *qnr* genes have also been isolated worldwide, and with the exception of *qnr*S are strongly associated with ESBL production[106]. Compared to other regions, both *qnr* and *aac(6')- Ib-cr* has been isolated with the highest frequency among *E. coli* in North America, Europe and South East Asia[176–180]. Both *qnr* and *aac(6')- Ib-cr* genes were recently described in *E. coli* isolates in Egypt[181]. The gene *aac(6')- Ib-cr* is the only PMQR determinant identified in South America and was identified in an urinary

E. coli isolate in Peru in 2005[182]. *Qep*A is the most recently described PMQR determinant and was first identified in an *E. coli* strain isolated from the urine of a hospitalized patient in Japan[183]. *Qep*A is less commonly associated with ESBL production and has been isolated in *E. coli* in North America, Europe, South East Asia, the Middle East and Egypt[175,179–181,184].

1.4.4.4 Clonal spread of uropathogenic *E. coli*

The epidemiology of the emergence of AMR among *E. coli* can generally be described as clonal or non-clonal in nature. The former refers to the propagation of a single resistant clone by the vertical transmission of resistance genes from parent bacteria to their offspring. Clonal spread is likely in a genetically homogenous population. These isolates cluster closely together when their relatedness is investigated by molecular techniques such as PFGE or MLST. In contrast, non-clonal spread occurs by the horizontal transmission of resistance determinants within an otherwise unrelated (heterogenous) population. This type of spread is facilitated by the presence of mobile genetic elements such as plasmids. Both epidemiological processes have been described in uropathogenic *E. coli*. However, the clonal spread of MDR strains has implications in community outbreak scenarios for interrupting transmission and reducing selective pressure for resistance[185]. This section will discuss four examples of MDR urinary *E. coli* clones identified in outbreak scenarios around the world[186,187].

1.4.4.4.1 *E. coli* O15:K52:H1

E. coli clonal group O15:K52:H1 was originally identified in South London during a twelve-month outbreak of community acquired UTIs between 1986-1987. In three

cases septicemia and additional infectious syndromes were identified and three patients died[188]. All isolates exhibited identical resistance phenotypes to ampicillin, streptomycin, tetracycline, sulfamethoxazole, trimethoprim and chloramphenicol. As a result, a local London hospital carried out screening of all blood and urine that was culture positive for *E. coli* between November 1986 and January 1988[189]. Of 25 urinary *E. coli* identified as O15, 80% expressed this resistance profile and 17 of these were temporally clustered in a four-month period. The proportion of serogroup O15 isolates fell gradually until, in June 1988, only 4.2% of urinary *E. coli* isolates belonged to this group[189]. Though this outbreak clustered together in time and space, additional investigations in Spain and North America indicate that this particular clone is widespread geographically. Despite possessing more diverse resistance profiles in these regions, members of this lineage retain a conserved and highly pathogenic virulence profile[190,191]. In fact, a more recent investigation of 199 *E. coli* isolates causing community acquired UTIs in Canada suggests that *E. coli* O15K2:H1 has re-emerged in Canada with a similar virulence profile and a recently acquired fluoroquinolone resistance phenotype[186].

1.4.4.4.2 *E. coli* O78:H10

A distinct strain, designated O78:H10, was found to be responsible for a more contained 1991 outbreak of community acquired UTIs in the greater Copenhagen area[192]. All isolates were lactose non fermenters and possessed identical phenotypes that included resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim. Twenty six percent of isolates examined over an eight-month period had an identical phenotype and belonged to the O78:H10 serotype[192]. All but one isolate originated from the urinary tract and 74% were community acquired.

Previous antibiotic exposure was a common risk factor among affected patients[192]. The clustering of this serotype in time and space points to the potential value of using phenotypic data as an initial screen to identify closely related MDR uropathogens by serotyping or more advanced molecular methods (PFGE, MLST).

1.4.4.4.3 Clonal Group A

A significant increase in the prevalence of SXT resistance among community acquired urinary *E. coli* in the late 1990s prompted multiple investigation into the epidemiological relationship of these isolates in the United States[193]. A single clonal group designated clonal group A (CGA) accounted for between 38% and 55% of SXT resistant community acquired UTIs in three geographically distinct communities across the U.S.[193]. In fact, during a four-month period, CGA accounted for roughly 9 and 11% of all UTIs in Minnesota and California respectively. What's more, CGA isolates were significantly more likely to exhibit MDR compared to non-CGA isolates[193]. Additional investigations in Colorado and multiple centers across the country found that CGA accounted for between 34% and 43% of SXT resistant UTIs in a similar time period[194,195]. What's more, molecular DNA fingerprinting and analysis of virulence factors found a highly conserved PFGE profile and virulence genotype among CGA isolates causing both pyelonephritis and cystitis across a vast geographical area[194]. It is likely that the clonal spread of this group made a significant contribution to the rapid emergence of SXT resistance observed in the U.S. at the beginning of the 21st century. More recently CGA has been identified among SXT resistant isolates in Montreal and, to a lesser extent, in the Calgary Health Region of Western Canada[186,196].

1.4.4.4.4 *E. coli* O25:H4:ST131

The most recent, widespread and extensively studied clonal group tied to community acquired uropathogenic *E. coli* was originally identified in the Northwest of England[197]. Members of the lineage ST131 (identified by MLST) were over represented (accounting for 59%) among uropathogens isolated in this region. What's more 98% of isolates belonging to ST131 were also found to be phenotypically resistant to ESCs[197]. Since it's identification, MDR urinary *E. coli* of the ST131 lineage (known more specifically as *E. coli* O25:H4:ST131), have been identified in India, Japan, Kuwait, Lebanon, Korea, Canada, the United States, Switzerland, France, Portugal, Spain, Croatia, the UK, Norway and Turkey[167,176,185,198–204].

Isolates belonging to ST131 tend to possess the ESBL allele CTX-M-15, are commonly fluoroquinolone resistance and are particularly pathogenic with respect to their virulence profile. Investigations into the virulence potential, AMR and epidemiological relatedness of ESBL and non ESBL producing *E. coli* in the U.S. found that the majority of isolates possessing ESBLs produced CTX-M 15 (58%) and that possession of CTX-M 15 was significantly associated with both fluoroquinolone resistance and ST131 membership[185]. Interestingly, even among isolates that did not produce ESBLs, members of ST131 were still significantly more likely to be fluoroquinolone resistant[185]. In the same study, the overall prevalence of ST131 was 16%, with isolates of this sequence type accounting for 67%–69% of ESC or fluoroquinolone resistant isolates, 55% of fluoroquinolones and SXT resistant isolates and 52% of MDR isolates[185]. ST131 *E. coli* also possess more diverse virulence profiles when compared with other resistant isolates[185]. Overall, members of this pandemic

lineage possess a unique combination of pathogenicity and MDR, with a high likelihood of both fluoroquinolone and/or ESC resistance[185]. A Canadian study investigated the prevalence of ST131 among ESBL producing *E. coli* collected from 11 different medical centers of which 78% were from UTIs and 62% were from community acquired infections[166]. Similar to the situation in the U.S., The majority of ESBLs identified were of the CTX-M type and CTX-M-15 (70%) was most common, though it ranged in frequency from 37% in British Columbia to 95% in Ontario[166]. ST131 comprised 46% of the population of ESBL producers, though this lineage ranged in prevalence across Canada from 21% in BC to 65% in Ontario[166]. The majority of ST131 *E. coli* (91%) produced CTX-M-15 and 69% were positive for the PMQR determinant *aac(6')-Ib-cr*[166]. Still another study investigated the prevalence of ST131 in urinary *E. coli* stratified for fluoroquinolone susceptibility. ST131 was overrepresented among fluoroquinolone resistant isolates, accounted for nearly half of the fluoroquinolone resistant population[186]. Interestingly, all but one ST131 isolate was susceptible to third generation cephalosporins and none possessed CTX-M-15[186]. The detection of fluoroquinolone resistant ST131 strains without CTX-M enzymes suggests that although this strain initially rose to prominence due to its association with CTX-M production, it is first and foremost a fluoroquinolone resistance pathogen that subsequently developed ESC resistance through the acquisition of CTX-M type enzymes. Thus, there is the potential for the future dissemination of CTX-M 15 within members of ST131 if frequent fluoroquinolone use continues in the treatment of uncomplicated cystitis across Canada. *E. coli* ST131 is a globally prevalent, MDR and particularly pathogenic cause of community acquired UTIs that appears to have disseminated by clonal spread in the early 21st century.

1.5 Antimicrobial resistance surveillance in canine urinary tract infections

1.5.1 Canadian Surveillance

Few studies have investigated the prevalence of resistance in canine urinary *E. coli* in Canada. Susceptibility results from canine urinary isolates submitted to a regional diagnostic laboratory in Western Canada were analyzed retrospectively[205]. This study included both non recurrent and recurrent infections, defined as more than one positive culture from the same patient during the study period. The panel of antimicrobials tested included ten drugs belonging to seven different classes[205]. *E. coli* was most frequently isolated (44.1%) followed by *Staphylococcus* spp (11.6%). Though resistance rates were not explicitly stated on a drug by drug basis, the authors found a significant increase in the number of antimicrobials to which recurrent *E. coli* were resistant over the study period[205]. In addition to being out of date, limitations of this study include the purely phenotypic nature of the results, and the employment of disk diffusion-based susceptibility testing. The use of molecular techniques could reveal Information about the mechanism and nature of resistance (chromosomal versus transmissible), while also identifying the emergence of globally prevalent resistance genes and strains. For example, *E. coli* producing CTX-M type ESBLs or strains belonging to ST131. The only study employing molecular techniques to investigate the prevalence and mechanisms of resistance in canine urinary *E. coli* in Western Canada revealed a remarkably susceptible population with 80% of isolates exhibiting pan susceptibility to all antimicrobials tested[122]. Reduced susceptibility to ampicillin (8.8%), and extremely low levels of quinolone resistance were identified with just 7% of isolates exhibiting resistance to nalidixic acid and <2% displaying ciprofloxacin resistance[122]. A single

CMY-2 type AmpC β-lactamase was identified[122]. Additional surveillance studies investigating resistance among canine uropathogens in Canada are needed to both inform prescribing practices and provide a baseline for future surveillance.

1.5.2 International Surveillance

Surveillance efforts for canine uropathogens are occurring on various scales across the globe. Often urinary *E. coli* are included as part of a larger investigation with respect to the infection site, host (most often canine and feline) and pathogens (*E. coli* among other Gram-positive and negative pathogens) included. This section summarizes the surveillance literature that exists for canine urinary *E. coli* around the world.

1.5.2.1 North America

In the United States pathogenic *E. coli* isolated from companion animals have been more extensively studied compared to Canada. Most studies have indiscriminately investigated clinical *E. coli* isolates from all extra-intestinal infection sites[206–208] in both cats and dogs[207,208]. These investigations indicate that most isolates are from canine infections (75-80%) with the majority obtained from the urinary tract (58-71%)[206–208]. Though the antimicrobial panels tested are not consistent across these studies, tetracyclines and β-lactams were the most common classes to which isolates were reported as resistant, especially the aminopenicillins amoxicillin and ampicillin (30-60%)[206–209]. In general, susceptibility to fluoroquinolones and aminoglycosides remained high (>80%) among pathogenic *E. coli*[206–208]. However, fluoroquinolone resistance was significantly associated with MDR in multiple investigations[207,208]. Though the definitions varied between studies, reported rates of MDR ranged from 21-52%[207–209]. One study

reported lower rates of MDR among urinary *E. coli* compared to *E. coli* isolated from non-urinary extra intestinal sites[208].

A single study focused exclusively on the susceptibility of canine uropathogens to commonly prescribed antimicrobials over a three-year period[209]. Among both uncomplicated and complicated infections, *E. coli* was the most frequently identified pathogen (52%). *E. coli* isolated from complicated UTIs were significantly more likely to be resistant to amoxicillin-clavulanate, ampicillin, chloramphenicol, doxycycline, enrofloxacin, ticarcillin-clavulanate and SXT when compared to uncomplicated UTIs[209]. The results of this and other longitudinal investigations suggest that, with the exception of tetracycline resistance, which increased significantly according to one study[207] the susceptibility of canine pathogenic *E. coli* to commonly prescribed oral antimicrobials remained stable over time[206,208,209].

1.5.2.2 South America

South America represents a significant gap in our knowledge of resistance among canine urinary pathogens. In a single study of Brazilian isolates, AMR and virulence was investigated among 43 *E. coli* isolated from both dogs (n=36) and cats (n=7)[210]. Of the agents tested, the frequency of resistance was highest to tetracycline (36%), ampicillin (37%), aminoglycosides (40%), and SXT (40%). Interestingly, all fluoroquinolone resistant isolates (16%) were also MDR and resistance to third generation cephalosporins was less than 5%[210]. Roughly half of the isolates were MDR to three or more antimicrobial classes. In general, resistance in this region is high with

less than 90% susceptibility reported to all antimicrobial classes with the exception of third generation cephalosporins and chloramphenicol[210].

1.5.2.3 Europe

Globally, Europe claims the most extensive literature investigating AMR among canine urinary *E. coli*. As of February 2018, a review of the literature reveals eleven investigations originating from nations that include Denmark, the Netherlands, Poland, Portugal, Sweden (n=2), Switzerland (n=2) and the United Kingdom (n=3). Regulations surrounding AMU in veterinary medicine are perhaps most progressive in Northern European countries such as Sweden and Norway where both AMR and AMU in companion animals are reported nationally[1]. Two Swedish studies conducted in 2005 and 2014, report relatively stable rates of AMR among canine urinary *E. coli* [211,212]. However, one investigation reported a more than 5% reduction in the prevalence of resistance to ampicillin and tetracycline when they compared urinary *E. coli* isolates collected in the periods 1991-1993 to those collected from 2002-2003[211]. Levels of resistance to all other antimicrobials remained stable over the ten year study period[211]. The more recent 2014 study reported similar results, including pan susceptibility rates of 79% among canine urinary *E. coli* isolated in Sweden between March and December 2009[212]. Reduced susceptibility was identified to ampicillin (87.9%) and amoxicillin-clavulanate (87.2%) and rates of MDR were astonishingly low at just 4%[212]. Studies originating out of Denmark and Poland investigated AMR among both canine and feline diagnostic *E. coli* isolates[213,214]. Compared to Sweden, Danish isolates were only slightly more resistant to common antimicrobials. Among 53 urinary *E. coli* isolates, the frequency of resistance was less than 20% across all agents tested with resistance to

sulfonamides (19.2%), tetracycline (19.2%) and ampicillin (15.4%) being reported most commonly[214]. In contrast, the frequency of resistance among canine diagnostic *E. coli* isolates in Poland was much higher. Though just 16.7% of isolates originated from the urinary tract, resistance exceeded 20% for all thirteen antimicrobials tested, including an alarming rate of amoxicillin resistance (~70%)[213]. Three studies have more specifically investigated β-lactamase production among urinary *E. coli* in Portugal, the Netherlands and Switzerland respectively[29,215,216]. High levels of resistance to first line drugs, including aminopenicillins, first generation cephalosporins and potentiated aminopenicillins were reported across all three studies. However, these isolates remain remarkably susceptible (>90%) to third generation cephalosporins[29,215,216]. In Switzerland, the frequency of AMR remained relatively stable over the ten-year period from 2003-2013. Among pathogenic *E. coli* isolated from companion animals, resistance rates of less than 10% were reported across all antimicrobials tested in one 2003 study[217]. Similarly in 2013, the majority of 107 urinary *E. coli* in Switzerland displayed pan susceptibility across all antimicrobials tested[216]. The most recent surveillance study out of the United Kingdom captured resistance among canine urinary isolates over a ten year period from 1999-2009 and concluded that there was no significant difference in the number of antimicrobials to which urinary *E. coli* were resistant over the study period[218]. Older longitudinal studies conducted in the UK from 1989 to 1997 included pathogenic canine *E. coli* isolated from dogs in community practice and hospital environments[219,220]. According to the authors, statistically significant changes in resistance were restricted to the β-lactams amoxicillin, and amoxicillin-clavulanate over

this time period, suggesting that rates of AMR have remained stable over the last decade[219,220].

1.5.2.4 Asia

In Asia, longitudinal UTI surveillance studies have been published from Japan, Iran and Taiwan[221–223]. While two of these studies focus exclusively on canine infections, including investigations into molecular mechanisms, the study out of Japan included strictly a phenotypic description of resistance among canine and feline pathogenic *E. coli*, isolated from both urinary and genital sites. However, the majority (63.4%) of isolates were obtained from canine UTIs[222]. Considering the geographic diversity of Asia including the isolated nature of Japan and Taiwan, it is perhaps not surprising that rates of resistance reported from these regions differ considerably. In Taiwan, the frequency of resistance was highest to oxytetracycline (60.5%) ampicillin (50%), amoxicillin (44.7%), nalidixic acid (38.6%), SXT (34.2%), chloramphenicol (31.6%) and doxycycline (28.9%) and ~50% of isolates exhibited MDR. The vast majority of isolates remained susceptible to the fluoroquinolones, aminoglycosides and amoxicillin-clavulanate with <10% resistance detected[223]. In Japan, resistance to at least one antimicrobial was reported in 62.5% of isolates and resistance to ampicillin (53%) and enrofloxacin (46%) was most prevalent. Approximately 53% of canine isolates were MDR and a single isolate obtained from a dog was pan resistant to all nine agents tested[222]. In Iran, urinary *E. coli* isolates were collected from healthy patients and those diagnosed clinically with UTIs. Overall, the highest rates of resistance were reported for gentamicin (95%), ampicillin (85%), amikacin (70%), amoxicillin (65%), and SXT (65%). Alarmingly, 100% of *E. coli* isolated from apparently health dogs harbored resistance to at least one

antimicrobial, while isolates from infected dogs demonstrated resistance to at least two agents[221].

1.5.2.5 Africa

Information regarding the prevalence of resistance among canine uropathogens on the African continent is limited. A single investigation conducted in South Africa in 2017 determined the susceptibility of canine urinary *E. coli* to fifteen drugs[224]. Alarming rates of resistance ranging from 65-100% were reported against penicillin, amoxicillin, cephalothin and doxycycline[224]. While still higher than most other regions, the frequency of resistance was much lower for enrofloxacin (16.7%) and orbifloxacin (21%), chloramphenicol (24.6%) and SXT (24.7%)[224]. None of the antimicrobial tested were more than 90% efficacious in vitro. What's more MDR, defined as resistance to at least one drug in more than three antimicrobial categories, was astonishingly common occurring in 98% of isolates[224].

1.5.2.6 Australia and New Zealand

Despite their relatively close proximity, canine urinary *E. coli* in Australia and New Zealand vary in their susceptibility to commonly prescribed antimicrobials. In New Zealand, a longitudinal study which took place from 2005-2012 reported significant increases in resistance to amoxicillin-clavulanate, cephalothin and enrofloxacin[225]. However, the frequency of resistance to clinically relevant antimicrobials remained below 10% for all agents with the exception of cephalothin (21.9%) in 2012[225].

In Australia, two studies have investigated pathogenic *E. coli* from canine and feline infections[21,226]. Among 883 *E. coli,* including 58.2% isolated from dogs, 61.3%

were pan susceptible to all antimicrobials tested. Among canine urinary E. coli specifically, resistance to β-lactams was most common, including cephalothin (95.4%), amoxicillin-clavulanate (28.6%) and ampicillin (26.8%)[226]. Susceptibility to fluoroquinolones, aminoglycosides and third generation cephalosporins was comparably high, while resistance to the carbapenem imipenem was not detected[226]. Older Australian data obtained in a case series investigating MDR extra-intestinal *E. co*li isolated from 37 dogs, found that all but one UTI resolved after treatment with amoxicillin-clavulanate or chloramphenicol[21]. These findings suggest that resistance to commonly prescribed antimicrobials, especially the β-lactam class, have increased substantially in Australia over the past decade.

In general, AMR among canine urinary *E. coli* across Europe (with the exception of Poland) and New Zealand have remained relatively low compared to other regions and are perhaps most comparable to those obtained in Canadian studies. On a global scale, relatively more European nations play host to long standing national surveillance programs, that include companion animal pathogens. The longitudinal nature of these efforts is particularly valuable for providing a reference to which the emergence of AMR in veterinary pathogens may be compared. In contrast, the frequency of resistance among canine urinary *E. coli* in Poland, Brazil, Asia and Africa is high compared to other regions and the prevalence of MDR is particularly alarming in these areas[213,222–224].

1.6 Antimicrobial resistance surveillance in veterinary medicine

Tracking the emergence and spread of AMR in veterinary pathogens is just one part of a complex surveillance system that considers both AMU and resistance in multiple host (human and veterinary) and microbial (commensal, clinical, zoonotic) populations. As of 2019, twenty countries currently play host to national surveillance systems that encompass AMU and/or AMR in veterinary medicine and these initiatives cover the Americas, Europe, Asia and Australia[1]. A summary of the scope of these programs is summarized in Table 1.6[1]. The resistance trends in human and (to a much lesser extent) canine urinary pathogens outlined thus far provide an excellent starting point for discussing the strengths and limitations of current and future surveillance initiatives in companion animal veterinary medicine.

Table 1.6: National AMR surveillance systems encompassing veterinary pathogens[1]

Program	Country	Status	Type	Scope	Sampling Type	Organisms	Funding
The Americas							
CIPARS[227]	Canada	Established 2002	AMU and AMR	Food animals, humans, food	AMR- clinical isolates from humans, isolates collected throughout food chain from animals, slaughterhouses and retail sources	*Salmonella* *Campylobacter* *E. coli*	Public health agency of Canada
NARMS[228]	United States	Established 1996	AMR	Food animals, humans, food	AMR- public health laboratories	*Salmonella* *Shigella* *E. coli 0157* *Vibrio* *Campylobacter*	FDA, USDA, CDC
COIPARS[229]	Colombia		AMR	Food animals, food	AMR- poultry throughout food chain	*Salmonella*	PHAC, Instituto Colombiano Agropecuario, WHO, Pan American Health Organization
Europe							
CODA-CERVA (Now SCIENSANO)[230]	Belgium	Veterinary monitoring established 2001	AMR	Food animals	AMR- On farm, healthy animals at slaughter	*Salmonella*, MRSA *Enterococcus*, *E. coli*	Federal public service for public health, food chain safety and environment
DANMAP[36]	Denmark	Established 1995	AMU and AMR	Food animals, companion animals, humans, food	AMU- pharmacies, veterinarians and feed mills AMR- Healthy production animals at slaughter,	*Salmonella*, *Campylobacter* *Clostridium difficile* *Enterococcus*, *E. coli*, ESBL producing Enterobacteriaceae	Ministry of health, ministry of science, innovation and higher education, ministry of food, agriculture and fisheries

FINRES-Vet[37]	Finland	Established 2002	AMU and AMR	Food animals	AMU- drug wholesalers AMR- clinical isolates and healthy animals at slaughter	*Salmonella* *Campylobacter* *Enterococcus* *E. coli*	Government agency
RESAPATH[38]	France	Established 1982	AMU and AMR	Food animals, companion animals, humans	AMU- sales data, all veterinary use AMR- Laboratory antibiogram data from diseased food and companion animals	*Salmonella* *Campylobacter* *Clostridium difficile* *Enterococcus* *E. coli*	Ministry of agriculture
BfT GERM-VET GERMAP[231]	Germany	Established 2001	AMU and AMR	Food animals, companion animals, humans	AMU- Sales data AMR- clinical data from laboratories	Range of organisms	Federal office of consumer protection and food safety,
Hungarian National System[232]	Hungary	Established 2001	AMR	Food animals	AMR- colon samples of food animals at slaughter	*E. coli* *Salmonella* *Campylobacter* *Enterococcus*	Ministry of Agriculture
ITAVARM[233]	Italy		AMU and AMR	Food animal, companion animals	AMU- all veterinary use AMR- clinical isolates, health animals at slaughter	*Salmonella* *E. coli* *Enterococcus* *Pasteurella* Staphylococci Streptococci *Brachyspira hyodysenteriae*	Government program
MARAN[234]	Netherlands		AMU and AMR	Food animals	AMU- drug sales from Dutch veterinary pharmaceutical industry AMR- Healthy animals at slaughter	*Salmonella* *Campylobacter* *Enterococcus* *E. coli* MRSA	Central veterinary institute within food and consumer product safety authority

NORM VET[39]	Norway	Established 2000	AMU and AMR	Food animals, companion animals, coordinates with human program (NORM)	AMU- drug wholesalers and feed mills AMR- active and passive laboratory data	*Salmonella* MRSA *E. coli*	Norwegian zoonosis centre at Norwegian Veterinary Institute
VAV Network (VISAVET)[235]	Spain	Established 1997	AMR	Food animals, companion animals	AMR- Zoonotic and commensal bacteria from slaughterhouses Pathogens from diagnostic laboratories	Healthy animals *E. coli* *Enterococcus* *Salmonella* *Campylobacter* Diseased *S. aureus* *E. coli* *Enterococcus* Food animals *E. coli* *Enterococcus* *Salmonella* *Campylobacter*	Ministry of environment, rural and marine affairs
SVARM[40]	Sweden	1992	AMU and AMR	Food animals, companion animals, coordinates with human program SWEDRES	AMU- Pharmacy data AMR- active and passive laboratory data	*Enterococcus* *E. coli* Various zoonotic	National veterinary institute Sweden
ARCH-VET[236]	Switzerland	Established 2002	AMU and AMR	Food animals, coordinates with human program ANRESIS	AMU- sales data AMR- Clinical isolates and healthy animals at slaughter	*Salmonella* *Campylobacter* MRSA *Enterococcus* *E. coli* ESBL producing Enterobacteriaceae	Government agency

UK-VARSS[237]/ SAVSNET[41]	United Kingdom		AMU and AMR	Food animals, companion animals,	AMU- sales data, survey data of prescribing practices AMR- clinical isolates, laboratory data	*Campylobacter* *E. coli* 25 species in total	Government agency under veterinary medicines directorate, British Small Animal Veterinary Association, University of Liverpool
Asia							
Domestic and veterinary use and drug residues in products of animal origin database retrieval system[238]	China		AMU and residues	Antimicrobials for veterinary use	AMU- active ingredient, type of disease, type of animal	N/A	Government program
NAMP[239]	Korea	Established 2009	AMR	Veterinary species, humans	Not specified	Not specified	Government program
JVARM[240]	Japan	Established 1999	AMU and AMR	Food animals	AMU- sales data, all veterinary use AMR- clinical laboratory isolates and healthy animals on farm	*Salmonella* *Campylobacter* *Enterococcus* *E. coli* *Actinobacillus pleuropneumoniae* *Pasteurella multocida* Streptococci *Mannheimia Haemolytica*	Ministry of Agriculture, Forestry and Fisheries
Australia and New Zealand							
Pilot program for AMR in bacteria of animal origin[241]	Australia	Pilot program 2003-2004	AMR	Food animals	AMR- healthy animals at slaughter	*E. coli* *Enterococcus* *Campylobacter*	Government program

CIPARS, Canada's National surveillance program, has been in place since 2002 and monitors both AMU and AMR in animals, humans and food. Upon closer inspection of the scope of this program, it becomes evident that resistance in veterinary pathogens of companion animal origin is not currently captured within the CIPARS framework[227]. In general, the utility of surveillance data is only as strong as the program used to obtain it. Key components in the development of a surveillance system include consideration for the program type, geographic range, program scope, specimen types and sampling programs, laboratory procedures and logistical issues such as the basis of participation, data reporting and funding/governance[1]. These factors provide the framework for discussing what a high-quality, disease specific AMR surveillance program for companion animal urinary pathogens might look like in Canada.

1.6.1 Program type

National surveillance systems vary substantially in their nature. Specifically, whether AMU and AMR are monitored in isolation or in an integrated fashion. For example, while countries like Denmark monitor AMU (VetStat) and AMR (DANMAP) under separate initiatives, France captures both under a single program (RESAPATH). Still other countries monitor only AMU (China) or AMR (Australia, U.S., Colombia, Belgium, Hungary, Spain and Korea). Variations in program type are often reflective of infrastructural differences between countries. For example, in Canada, CIPARS is able to track the wholesale of drugs sold for use in animals, however, they are unable to report more specifically on veterinary AMU as there is no centralized body for mandatory reporting[1,30,242]. Furthermore, Canadian veterinarians' practice of both prescribing and dispensing antimicrobials at the individual practice level, at times

prescribing generic formulations from human pharmacies, or use of antimicrobials in an extra label manner, makes this information particularly difficult to capture in surveillance programs[242]. This is in contrast to European nations like Sweden and Denmark, where all veterinary AMU is by prescription only and is therefore nationally reported[1,242]. An ideal and truly epidemiology-based system would successfully track and integrate information regarding general and disease specific AMU and resistance in humans, animals and environmental sources including food.

1.6.2 Geographic range

Surveillance systems vary from local to global in their geographic range. For example, the Global Antimicrobial Resistance Surveillance System (GLASS) launched by WHO in 2015 seeks to develop a standardised approach to the collection, processing and sharing of AMR surveillance data across the globe[243]. While global surveillance efforts are informative for making comparisons between regions, local or institution specific data is required for informing prescribing practices[244]. For example, the geographic extent and diversity of Canada necessitates the implementation of regional programs to establish local estimates of AMU and AMR in veterinary pathogens[1]. Such programs provide invaluable epidemiological information to advise local AMU and to describe the emergence of AMR[50]. In fact, as discussed within the context of human urinary pathogens, there is strong evidence for significant geographic variability in resistance, further supporting the need for region specific surveillance efforts in Canada[30,68,245].

1.6.3 Program scope

Program scope generally refers to the hosts (healthy or diseased companion animals, food animals, humans, food), bacteria (zoonotic, commensal, pathogenic) and antimicrobial agents included in a surveillance system[1]. In fact, the scope of a surveillance program is greatly influenced by regional differences in production systems, disease pressure, resources for disease control and prevention and access to antimicrobials[242]. For example, as a developed nation with relatively low disease pressure and access to diagnostic resources, CIPARS tracks resistance in healthy animals, diseased animals, diseased humans and retail food sources including beef and chicken. However, the scope of this program is largely geared towards identifying human health hazards such that the only veterinary species monitored are production animals raised for human consumption including poultry (chickens and turkeys), pigs, beef cattle, and to some extent, horses. Companion animals, on the other hand, are not included[1]. This is in contrast to European nations, including France, Germany, Italy, Norway, Spain, and Sweden who monitor AMR in clinical isolates from companion animals[1].

With respect to bacterial species, CIPARS monitors commensal and pathogenic enteric organisms from food animals with the potential for food borne transmission including[227]:

- *Salmonella* spp: Clinical isolates from humans, horses, turkeys, chickens, pigs and beef cattle, abattoir samples from chickens and pigs, on farm samples from beef cattle, chickens, turkeys and pigs, retail chicken and turkey samples

- *Campylobacter* spp.: Abattoir samples from beef cattle, chickens and pigs, on farm samples from beef cattle, chickens and turkeys, retail chicken and turkey samples
- *E. coli*: Abattoir samples from beef cattle chickens and pigs, on farm samples from beef cattle, chickens, turkeys and pigs, retail chicken, turkey and beef samples

While this restricted scope is not surprising considering a major objective of CIPARS is to assess the public health impact of AMU and AMR in humans and animals, veterinary guidelines identify canine UTIs caused by *E. coli* and skin infections caused by *Staphylococcus* spp. as important targets worthy of surveillance[49]. Because these companion animal pathogens are not addressed by CIPARS, monitoring their susceptibility relies on regional investigations if information about the prevalence of resistance to clinically important antimicrobials is to be obtained[49].

A common criticism of CIPARS is its lack of clearly articulated goals or targets for success[242]. This is a common issue with surveillance studies as most often they are descriptive in nature, successfully identifying phenotypic resistance but failing to provide a molecular explanation or a practical context within which the results can be interoperated or applied. In fact, there is emerging evidence in human medicine that AMR surveillance has a role to play in disease based antimicrobial stewardship programs[246]. For example, with respect to reducing AMU and improving management of asymptomatic bacteriuria and UTIs, a combination of guideline implementation and education on local uropathogen resistance rates improved prescribing patterns and management of these infections in multiple clinical settings[246]. In a reciprocal manner,

surveillance initiatives could use guidelines published by organizations like the IDSA, or ISCAID to provide scope and focus for surveillance with respect to defining pathogens and antimicrobials of clinical interest. Clearly defined objectives, whether they be to establish a baseline for future surveillance studies (phenotypic), to detect the emergence of resistance in a region (molecular) or to inform treatment decisions (phenotypic in the context of clinical guidelines) drastically change the inclusion criteria and scope of surveillance studies.

1.6.4 Specimen type and sampling procedures

Data collection in surveillance systems can be active; involving the application of some systematic search tool to identify cases, or passive; based on voluntary or routine reporting systems[149]. In both cases, selection and sampling bias are imbedded in the nature of surveillance studies. With respect to AMR, passive, or convenience sampling, can either involve laboratory isolates or laboratory susceptibility data[242]. In most cases these studies are also retrospective in nature such that the amount of clinical data, including patient demographics, case definitions and clinical outcomes is limited[242]. For example, regarding sampling procedures for *Salmonella*; CIPARS' reliance on passively acquired laboratory isolates resulted in significant sampling bias such that greater than 70% of their isolates originated from a single province over one year[242]. The Danish national surveillance program DANMAP, on the other hand, used a form of "pseudorandom" selection of isolates from three different national centers for their *Salmonella* surveillance[242]. In the case of urinary isolates, resistance patterns captured by laboratory susceptibility data most accurately reflect complicated or recurrent UTIs since empirical treatment is common and culture is unusual unless initial treatment has

failed[50]. In general, both sampling and selection bias can be mitigated by increasing the amount of epidemiological data collected per isolate, as occurs most often in prospective studies[242]. What's more the application of strict inclusion criteria regarding specimen type, and method of collection, for example, further mitigate the inherent biases that often plague surveillance data.

1.6.5 Laboratory procedures

Laboratory procedures regarding testing and interpretation are inconsistent across the surveillance literature and are often based on the standard protocols of the submitting laboratory rather than a programmatic standard. Definitions of susceptible, intermediate and resistant used to interoperate susceptibility results are highly variable across the literature, with the intermediate category inconsistently categorized into one of the other groups. While standardized quality control strains and interoperative breakpoints exist, they vary according to region; European Committee on Antimicrobial Susceptibility testing, (EUCAST) versus CLSI, host; human versus veterinary and infection site; urine, versus blood or bladder, for example. What's more definitions of MDR; a very commonly reported parameter in microbiology, varies based on different interpretations of what constitutes an antimicrobial class.

1.6.6 Logistical issues

Participation incentives, funding, data analysis and reporting are just some of the logistical issues baring the implementation of effective AMR surveillance programs. Surveillance often requires participation from multiple stakeholders including producers, microbiology laboratories, regulatory bodies and pharmaceutical companies.

Participation may be based on mandatory, randomised or voluntary reporting of AMU or AMR data. The associated pros and cons of different schemes as well as legal issues regarding prescription only antimicrobials need to be considered[1]. Funding and reporting often go hand in hand, with funding agencies requiring data collection and reporting at regular intervals. In fact, isolate collection for surveillance often precedes microbial analysis in the face of insufficient funds[242]. Funding sources vary considerably from pharmaceutical companies (MYSTIC), to government agencies (CIPARS) and academic institutions (SAVSNET). With respect to funding sources, the potential for conflict of interests, particularly when pharmaceutical companies are involved, should be identified[242].

1.7 Rationale

The identification of urinary *E. coli* as an important target for surveillance efforts is a relatively recent phenomena in veterinary medicine[49]. The regularity with which this pathogen is isolated in dogs, warrants an investigation into the frequency and mechanisms of resistance in this population. The reviewed literature indicates that rates of resistance in canine isolates are low compared to synonymous human infections, though there is significant geographic variation[68,94,151,154,247]. For example, resistance is more prevalent in Asian and African countries, when compared to Canada and northern European countries such as Sweden[122,205,212,221–224]. In most cases surveillance investigations are descriptive and retrospective in nature, relaying the results of qualitative susceptibility testing without further investigation of the genetic basis of

resistance. The identification of phenotypic AMR, while sufficient for clinical purposes, provides an incomplete description of how and why resistance occurs. Molecular techniques allow for more extensive investigations into the mechanism of resistance, and by extension, provide important epidemiological information about the emergence and spread of resistance genes. What's more, the inclusion criteria, sampling and submission procedures, method of susceptibility testing, panel of antimicrobials included; and definitions of clinical microbiological parameters such as susceptible, resistant and MDR are highly variable across the literature, making generalizing and comparing results difficult. These surveillance studies also tend to be passive, relying on susceptibility data or diagnostic submissions to regional laboratories. Convenience sampling in this way introduces bias in surveillance literature since laboratory submissions typically represent scenarios where empirical treatment has failed[50]. This study aims to develop a pathogen (*E. coli*) and disease specific (canine UTIs) surveillance program within a specified geographic area (Saskatchewan) in order to fill the knowledge gap that currently exists regarding the state of AMR in canine urinary *E. coli* in this region. Strict inclusion criteria and robust methods, including standardized antimicrobial panels and interoperative criteria, will be applied to gather accurate, local resistance data that may be used to establish a baseline for future surveillance, in addition to being used by local veterinarians to inform prescribing practices for these common infections.

1.8 Objectives

1. To describe the phenotypic resistance profiles of canine urinary *E. coli* in Western Canada during a four-year surveillance period

2. To characterize the molecular mechanism(s) of antimicrobial resistance among *E. coli* causing canine urinary tract infections in Western Canada during a four-year surveillance period

3. To describe fosfomycin susceptibility and mechanisms of resistance among *E. coli* and *S. pseudintermedius* isolated from canine UTIs in Saskatchewan

2. CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE AMONG *E. COLI* CAUSING CANINE URINARY TRACT INFECTIONS: PASSIVE SURVEILLANCE OF LABORATORY ISOLATES IN SASKATOON, CANADA 2014-2018

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2.1 Abstract

Background: Urinary tract infections are common in veterinary medicine. The urinary tract is the most common source of MDR *E. coli* in canine patients and a treatment challenge for clinicians. Surveillance to detect the emergence of AMR in companion animal pathogens is lacking in Western Canada.

Methods: From November 2014 to 2018, 516 non duplicate canine urinary *E. coli* isolates were collected from a diagnostic laboratory. Susceptibility testing was performed against a panel of 14 antimicrobials belonging to 7 different drug classes and isolates with appropriate phenotypes were screened for production of ESBLs, AmpC β-lactamases and PMQR genes. Epidemiological relationships were assessed by MLST.

Results: 80.2% (n=516) of isolates were susceptible to all antimicrobials tested. There was no significant increase in the proportion of isolates resistant to any of the tested antimicrobials during the study period. However, resistance to sulfisoxazole and SXT decreased significantly between years three and four (p=0.0073) and three and five (p=0.0066) respectively. Resistance to ampicillin was most common (14.9%). Overall, twelve isolates harbored CMY-2 type AmpC β-lactamases, and seven produced CTX-M type ESBLs. A single isolate was found to harbour the *aac(6')- Ib-cr* PMQR gene. The *qnr* and *qep*A determinants were not detected. A single isolate belonging to the pandemic lineage ST131 was identified.

Conclusion: Canine urinary *E. coli* in Saskatchewan remain susceptible to first line therapies, though resistance, particularly to the aminopenicillins, warrants monitoring. This is the first description of *E. coli* ST131 from a companion animal in Canada.

2.2 Introduction

AMR has become a common concern of animal and human health care professionals. MDR infections increase the morbidity and mortality of patients and result in increased costs to the client and the health care system[49,248]. In dogs, the urinary tract has been implicated as the most common extra intestinal site for MDR *E. coli*[21]. What's more, the close contact between humans and their pets has implications for the sharing of resistant commensal and pathogenic bacteria between species. While the exact role of dogs in this transmission cycle has not been adequately studied, evidence of "strain sharing" has been demonstrated with *Clostridium difficile* and MRSA[24–26,28]. Similarly, multiple studies have shown support for the sharing of *E. coli* between dogs and humans through the simultaneous isolation of MDR strains from cohabitating humans and their canine companions in both hospital and home environments[22,23].

Antimicrobials are commonly used in companion animal veterinary practice. One study in the United Kingdom estimated that more than one third of consults with canine patients resulted in the prescription of antimicrobials[42]. Uncomplicated or sporadic UTIs occur in approximately 14% of dogs that visit a veterinarian in their lifetime, representing a frequent reason for the prescription of antimicrobials[53]. Similar to the case in humans, the Gram-negative bacterium *E. co*li is the most frequent pathogen in UTI cases, accounting for upwards of 50% of positive canine urine cultures[52,56,68,249]. According to ISCAID, empirical treatment options include members of the β-lactam class; such as amoxicillin, or the potentiated sulfonamide, SXT[50,54,58]. Alternative therapies with a broader spectrum of activity, such as fluoroquinolones (enrofloxacin, ciprofloxacin), chloramphenicol, doxycycline, fosfomycin and nitrofurantoin should be reserved for

cases where laboratory results indicate a lack of susceptibility to empirical treatments. In general, it is considered unnecessary to prescribe these agents in cases of uncomplicated cystitis given their relative importance in treating human infections, coupled with the increasing frequency of resistance[54].

From a clinical perspective, the most troubling resistance mechanism in canine urinary *E. coli*, include ESBLs, Class C (AmpC) β-lactamases, and PMQR genes[103]. ESBLs are capable of hydrolyzing even those extended-spectrum β-lactams that had been modified to confer resistance to the first-generation enzymes. The first ESBL identified in an animal was isolated from the feces of a healthy laboratory dog in Japan[250]. In general, SHV, TEM and CTX-M type ESBLs are among the most common, however since the beginning of the 21st century, CTX-M type enzymes have become most prevalent[76,110,117]. Currently, the most widely distributed CTX-M alleles are CTX-M-1, -15 and -14, having been isolated from both healthy and diseased companion animals[103]. While CTX-M-1 and CTX-M-15 predominate in Europe, in North America, CTX-M-15 is most commonly encountered, particularly among companion animals suffering from cystitis[29,127,128,135,138,216,251–257]. In Asia CTX-M-14 is the dominant enzyme type, however, additional ESBLs, including SHV-12 have been identified in companion animals in Spain, Germany, China and the U.S.[136,253,255,258]. AmpC β-lactamases confer resistance to cephamycins (cefoxitin for example) and "classic" β-lactamase inhibitors, in addition to ESCs. Most often, AmpC β-lactamase producing *E.coli* acquire these enzymes on plasmids[119]. CMY-2 is the most common type detected in companion animals, having been identified in dogs from Japan, Denmark and Canada[120–122]. Fluoroquinolones are a broad-spectrum antimicrobial class that exert their mechanism

of action by interfering with DNA replication[49]. Originally, acquired resistance to fluoroquinolones was thought to occur strictly through chromosomal mutation in the genes *gyr*A and *par*C that encode the drug target[45,131]. However, the first report of PMQR occurred in the late 1990s[132]. To date, three types of transmissible quinolone resistance genes have been reported. The target protecting proteins (*qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S); the inactivating enzyme *aac(6')- Ib-cr*, and the efflux pumps *qep*A and *oqx*AB[45]. In companion animals the gene *aac(6')- Ib-cr* appears to be the most widespread, having been identified in *E. coli* from both healthy and diseased companion animals across Europe, Australia, Asia and the U.S.[135–139,142,143]. The tendency of the *aac(6')- Ib-cr* gene to co-locate on plasmids harbouring ESBLs is of particular concern, as it provides the framework for the emergence of MDR. In Canada, the efflux pump QepA and the Aac(6′)-Ib-cr enzyme have been much more commonly reported than the Qnr proteins among urinary *E. coli* isolated from humans[173,175,259]. However, we know of no studies documenting either of these PMQR determinants in companion animal *E. coli* in Canada.

The emergence of transmissible AMR among canine urinary *E. coli* in other regions warrants an investigation into the prevalence and mechanisms of resistance in this pathogen population in Canada. In fact, various consensus statements in veterinary medicine call for more prudent use of antimicrobials by veterinarians and identify urinary *E. coli* as an important target for surveillance studies[15,49,50]. Unfortunately, national surveillance programs in Canada do not address AMR in companion animals, focusing instead on food animal pathogens with the potential for foodborne transmission[1].

Where surveillance literature exists for canine uropathogens, the results indicate significant geographic variation in the frequency of resistance. For example, resistance is more prevalent in Asian and African countries, compared to Canada and northern European countries such as Sweden[122,205,212,221–224]. Previous work out of Saskatchewan detected a particularly low frequency of resistance (~80% pan susceptibility) among canine urinary *E. coli*[122]. These baseline findings provide a unique opportunity to detect the emergence of clinically relevant resistance determinates in this region. This study aims to describe the frequency and mechanisms of AMR among *E. coli* causing canine UTIs in Western Canada during a four-year surveillance period.

2.3 Materials and Methods

2.3.1 Microbiological analysis and inclusion criteria

Canine diagnostic samples submitted to Prairie Diagnostic Services (PDS) in Saskatoon, SK were collected from October 2014-2018. Samples were obtained as pure cultures plated on 5% Columbia blood agar on a weekly basis. Initial bacterial identification was performed at PDS by biochemical testing; including indole, triple sugar iron (TSI) agar, urea and citrate (2014-2015); and matrix-based assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy (2015-2018). In cases of suspicious colony morphology or contamination, bacterial identity was confirmed based on lactose fermentation on MacConkey agar and the spot indole test. Rarely, colony morphology and biochemical tests were insufficient for species identification. In these cases, PCR was employed to amplify a universal gene; *16S rRNA* or *cpn*60[260,261]. All isolates were saved for analysis in tryptic soy broth (TSB) plus 15% glycerol at -80°C. All canine urinary submissions were initially included.

In order to be included in the study, isolates had to be confirmed as first-time submissions of canine urine with a positive *E. coli* culture from one of the four western Canadian provinces. Data extracted from the PDS database to confirm the identity of repeat samples include microbiological information, such as laboratory analytical procedure, bacterial species and specimen type; and patient information, including animal name, species, breed, age, client surname and case number, where available. Geographic information regarding the origin of each submission (client province of

origin), was recorded to determine the extent to which our study covered the Western Canadian provinces.

2.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed by the broth microdilution technique using the Sensititre system. Briefly, standard 0.5 McFarland suspensions were prepared in commercially obtained sterile water (Thermo Fisher Scientific) using a fresh overnight culture plated on 5% Columbia agar. A 30 μL volume of the bacterial suspension was transferred to Sensititre Mueller-Hinton broth. Following the application of a dosing head, 50 μLs was auto inoculated onto 96 well plates containing two-fold serial dilutions of various antimicrobials. Plates were sealed and following overnight incubation at 35°C, growth was observed and recorded manually on plate schematics obtained on the manufacturer website. We employed the Gram-negative panel (CMV3AGNF/CMV4AGNF) used by national surveillance studies targeting food borne pathogens in Canada and the U.S., which includes 14 antimicrobials belonging to 7 different classes (Table 2.1). Agents tested include ampicillin, amoxicillin-clavulanate, cefoxitin, ceftriaxone, ceftiofur, nalidixic acid, ciprofloxacin, gentamicin, tetracycline, chloramphenicol, sulfisoxazole, trimethoprim-sulfamethoxazole, and azithromycin. This particular panel was updated to include the carbapenem meropenem, in place of ceftiofur in 2017. To ensure the AST system performed as expected, quality control strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were run in parallel with test isolates. Results were interoperated according to breakpoints published by CLSI in their M100 (29th ed.) document[262]. These values have taken into account microbiological, pharmacologic and clinical data

to synthesize the efficacy of certain drug-bug combinations for the infection under investigation and are summarized in Table 2.1. Since some antimicrobial agents tend to concentrate in the urine significantly above levels attainable in the blood, where available we utilized urine specific breakpoints[209]. In addition to resistance to these drugs, we also report rates of MDR which was defined as resistance to at least one antimicrobial in three or more classes[263]. For the purposes of this study, we consider all β-lactams as one class.

Table 2.1: Antimicrobials, concentrations and breakpoints used for susceptibility testing of *E. coli* (CMV3AGNF/CMV4AGNF)[262]

Antimicrobial	Concentrations (μg/mL)	CLSI breakpoint (μg/mL)
CMV3AGNF/CMV4AGNF		
Cefoxitin (FOX)	0.5-32	≥32
[1]Azithromycin (AZI)	0.12-16	≥32
Chloramphenicol (CHL)	2-32	≥32
Tetracycline (TET)	4-32	≥16
Ceftriaxone (AXO)	0.25-64	≥4
[2]Amoxicillin-clavulanate (2:1 ratio) (AUG2)	1/0.5-32/16	≥32/16
Ciprofloxacin (CIP)	0.015-4	≥4
Gentamicin (GEN)	0.25-16	≥16
Nalidixic Acid (NAL)	0.5-32	≥32
Ceftiofur (XNL)	0.12-8	≥8
[3]Meropenem (MER)	0.06-4	≥4
Sulfisoxazole (FIS)	16-256	≥512
Trimethoprim-sulfamethoxazole (SXT)	0.12/2.38-4/76	≥4/76
Ampicillin (AMP)	1-32	≥32

[1]Concentrations included for azithromycin AST by broth microdilution were changed to include 32 μg/mL in the most recent version of the NARMS panel (CMV4AGNF) released in 2017.

[2]Amoxicillin-clavulanate contains amoxicillin and clavulanic acid in a 2:1 ratio

[3]Ceftiofur was replaced with Meropenem in the most recent version of the NARMS Sensititre panel (CMV4AGNF) released in 2017.

2.3.3 Detection of resistance genes

DNA was prepared for PCR using a heat based crude extraction method. From fresh overnight culture, four to five colonies were transferred to 200 μL of sterile water and heated at 95°C for ten minutes. The resulting suspension was spun down and the supernatant removed and stored at -20°C for future use as a DNA template. If resistance phenotypes involved third generation cephalosporins, cephamycins or fluoroquinolones, isolates were sequentially screened for various resistance genes by PCR. General PCR conditions included denaturation at 94°C for 6 minutes followed by 30-35 cycles of denaturation (94°C), annealing (various temperatures), and extension (72°C) for 1 minute each and a final extension step for 10 minutes at 72°C. All PCR reactions included a no template negative control and a positive control containing DNA from an isolate previously determined to be positive for the gene of interest. Primers and associated annealing temperatures utilized for all reactions are shown in Table 2.2.

2.3.3.1 Broad spectrum β-lactamases

All third-generation cephalosporin (ceftriaxone or ceftiofur) resistant isolates were initially screened using universal primers for CTX-M type enzymes. Any positive isolates were sequentially screened for CTX-M groups 1, 2, 8 and 9. Isolates were additionally screened for the SHV and TEM type ESBLs. Cephamycin (cefoxitin) resistant isolates were screened for CMY-2 type AmpC β-lactamase production. All primers and PCR conditions are outlined in Table 2.2.

2.3.3.2 Plasmid mediated quinolone resistance determinants

Nalidixic acid and ciprofloxacin resistant isolates were screened for each of the PMQR determinants; *qnr*A, *qnr*B, *qnr*S, *aac(6')-Ib-cr*, and *qep*A using primers summarized in Table 2.2.

Table 2.2: Primers and PCR conditions

Name	Nucleotide sequence (5'-3')	Amplicon size	Annealing Temperature (°C)	Reference
16S (1476/1478)	GAGTTTGATCCTGGCTCAG	500	60°C	[261]
	G(TA)ATTACCGCGGC(TG)GCTG			
AAC(6')-IB-CR	CATTGTAGCACGTGTGTA	482	67°C	[264]
	CATTGTAGCATGCG'I"I'GAAG			
ADK	CGGTGTGT[AG]CAAGGCCC	583	54°C	[265]
	ACGGCTACCTTGTTACGACTT			
CMY-2	AGAAAGGAGGTGATCCAGCC	1000	68°C	[266]
	GCTTTTCAAGAATGCGCCAGG			
CPN60 (1594/1595)	CGCCAGGGTTTTCCCAGTCACGACGACGTCGCCGGTGACGGCACCACCAC	700	57°C	[260]
	AGCGGATAACAATTTCACACAGGACGACGGTCGCCGAAGCCCGGGGCCTT			
CTX-M G1	GTTGTTAATTCGTCTCTTCC	700	61°C	[267,268]
	AGTTTCCCCATTCCGTTTC			
CTX-M G2	ACTCAGAGCATTCGCCGCTCA	1000	58.3°C	[268]
	TTATTGCATCAGAAACCGTG			
CTX-M G8	CGCTTTGCCATGTGCAGCACC	307	64°C	[269]
	GCTCAGTACGATCGAGCC			
CTX-M G9	GACCGTATTGGGAGTTTGAG	600	48°C	[270,271]
	ATCTGATCCTTCAACTCAGC			
CTX-M U	ATGTGCAGYACCAGTAARGTKATGGC	593	61°C	[267]
	TGGGTRAARTARGTSACCAGAAYCAGCGG			
FUMC	TCACAGGTCGCCAGCGCTTC	806	54°C	[265]
	GTACGCAGCGAAAAAGATTC			
GYRB	TCGGCGACACGGATGACGGC	911	61°C	[265]
	ATCAGGCCTTCACGCGCATC			
ICD	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878	54°C	[265]
	GGACGCAGCAGGATCTGTT			
MDH	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932	54°C	[265]
	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT			
PURA	CGCGCTGATGAAAGAGATGA	816	54°C	[265]
	CATACGGTAAGCCACGCAGA			
QEPA	GCAGGTCCAGCAGCGGGTAG	199	60°C	[272]
	CTTCCTGCCCGAGTATCGTG			
QNRA	ATTTCTCACGCCAGGATTTG	516	53.4°C	[273]
	GATCGGCAAAGGTTAGGTCA			

QNRB	GATCGTGAAAGCCAGAAAGG	469	67°C	
	ACGATGCCTGGTAGTTGTCC			
QNRS	ACGACATTCGTCAACTGCAA	417	53°C	
	TAAATTGGCACCCTGTAGGC			
RECA	TCTCGATCAGCTTCTCTTTT	780	61°C	[265]
	CGCATTCGCTTTACCCTGACC			
SHV	TTATCTCCCTGTTAGCCACC	797	68°C	[274]
	GATTTGCTGATTTCGCTCGG			
TEM	GCGGAACCCCTATTTG	964	58.3°C	[275]
	ACCAATGCTTAATCAGTGAG			

2.3.3.3 PCR purification and sequencing

Following PCR, amplicons were further purified using the EZ-10 Spin Column PCR Purification Kit, (Bio Basic Canada Inc) or the ExoProStar enzymatic PCR and sequence reaction clean up kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. DNA sequencing was performed by Macrogen Inc. Obtained sequences were compared to reference genes published in the National Center for Biotechnology Information (NCBI) and Comprehensive Antibiotic Resistance Databases (CARD) using the Basic Local Alignment Search Tool (BLAST) algorithm.

2.3.4 Molecular epidemiology of ESBL and PMQR producing isolates

To determine epidemiological relationships between ESBL and PMQR producers, we utilize the MLST scheme for *E. coli* described by the Warwick Institute, (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) as this scheme has the greatest agreement with whole genome sequencing (WGS) techniques[162,265,276,277]. In short, for each isolate, seven different housekeeping genes (*adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A and *rec*A) were amplified by PCR and the purified products were sequenced. Obtained sequences were compared to an MLST database (pubMLST), which assigns each allelic profile to a sequence type (for example, ST131). Primers and annealing temperatures used for these reactions are outlined in Table 2.2.

2.3.5 Statistical Analysis

Susceptibility data was grouped into susceptible and resistant for each antimicrobial across the four years of study. The relationship between the proportion of resistant and susceptible isolates to a given antimicrobial and the year of submission was assessed using the Fisher's exact test for low cell frequencies with Bonferroni's correction ($p<0.01$). The change in the proportion of MDR and pan susceptible isolates over time was examined using the Fisher's exact test with Bonferroni correction ($p<0.01$). The relationship between β-lactamase production and the phenotypes MDR and fluoroquinolone resistance were also assessed using a Fisher's exact test ($p<0.01$).

2.4 Results

2.4.1 Microbiological analysis and inclusion criteria

From November 1st, 2014 to October 31st, 2018 710 *E. coli* isolates were obtained from PDS. Non urine (n=28), non-canine (n=24), non-*E*. *coli* (n=15) and repeat (n=127) isolates were excluded as shown in Table 2.3, leaving a total of 516 non duplicate canine urinary *E. coli* isolates. As shown in Table 2.4, 92.8% (n=479) of isolates originated from Saskatchewan, while the remaining 7% was split equally between Manitoba (n=18) and Alberta (n=18). A single isolate was submitted from British Columbia (n=1).

Table 2.3: An overview of excluded isolates (n=194)

Exclusion Criteria	**Number of Isolates**
Not urine	28
Ear/skin swab	10
Feces	3
Other	15
Not canine	24
Equine	1
Feline	21
Porcine	2
Not *E. coli*	15
Citrobacter spp.	3
Enterobacter spp.	1
Enterococcus spp.	6
Pseudomonas spp.	1
Staphylococcus pseudintermedius	4
Repeat submissions	127

Table 2.4: Geographic distribution of isolates (n=516)

Province	Year 1	Year 2	Year 3	Year 4	Total
Alberta (n=18)	3	6	2	7	3.5%
British Columbia (n=1)	0	0	0	1	0.2%
Manitoba (n=18)	3	1	6	8	3.5%
Saskatchewan (n=479)	80	139	124	136	92.8%

2.4.2 Antimicrobial susceptibility testing

There was no statistically significant increase in the frequency of resistance to any of the fourteen antimicrobials tested across the study period (Figure 2.1 and 2.2). There was a statistically significant decrease in the proportion of isolates resistant to sulfisoxazole and SXT observed during the study period ($p<0.01$) between years three and four ($p=0.0073$) and years three and five ($p=0.0066$) respectively. Overall, 80.2% of isolates were pan susceptible to all antimicrobials tested, while 6.0% exhibited MDR to at least three different antimicrobial classes. There was no change in the frequency of pan-susceptibility or MDR over the study period. Susceptibility remained above 90% for all agents tested except ampicillin (85%). After ampicillin, resistance was most common to tetracycline (7.7%), sulfisoxazole (7.3%), nalidixic acid (5.7%), cefoxitin (5.7%) and amoxicillin-clavulanate (5.5%). The frequency of resistance for the remaining antimicrobials was less than 5%. Meropenem resistance was not detected. The majority of isolates resistant to a single antimicrobial class were resistant to β-lactams (73%). The 31 MDR isolates displayed 26 different resistance profiles as shown in Table 2.5. Of the MDR isolates, the majority of phenotypes included resistance to β-lactams (93.5%) and/or tetracyclines (80.6%). A single isolate was found to be resistant to all seven antimicrobial classes included on the panel.

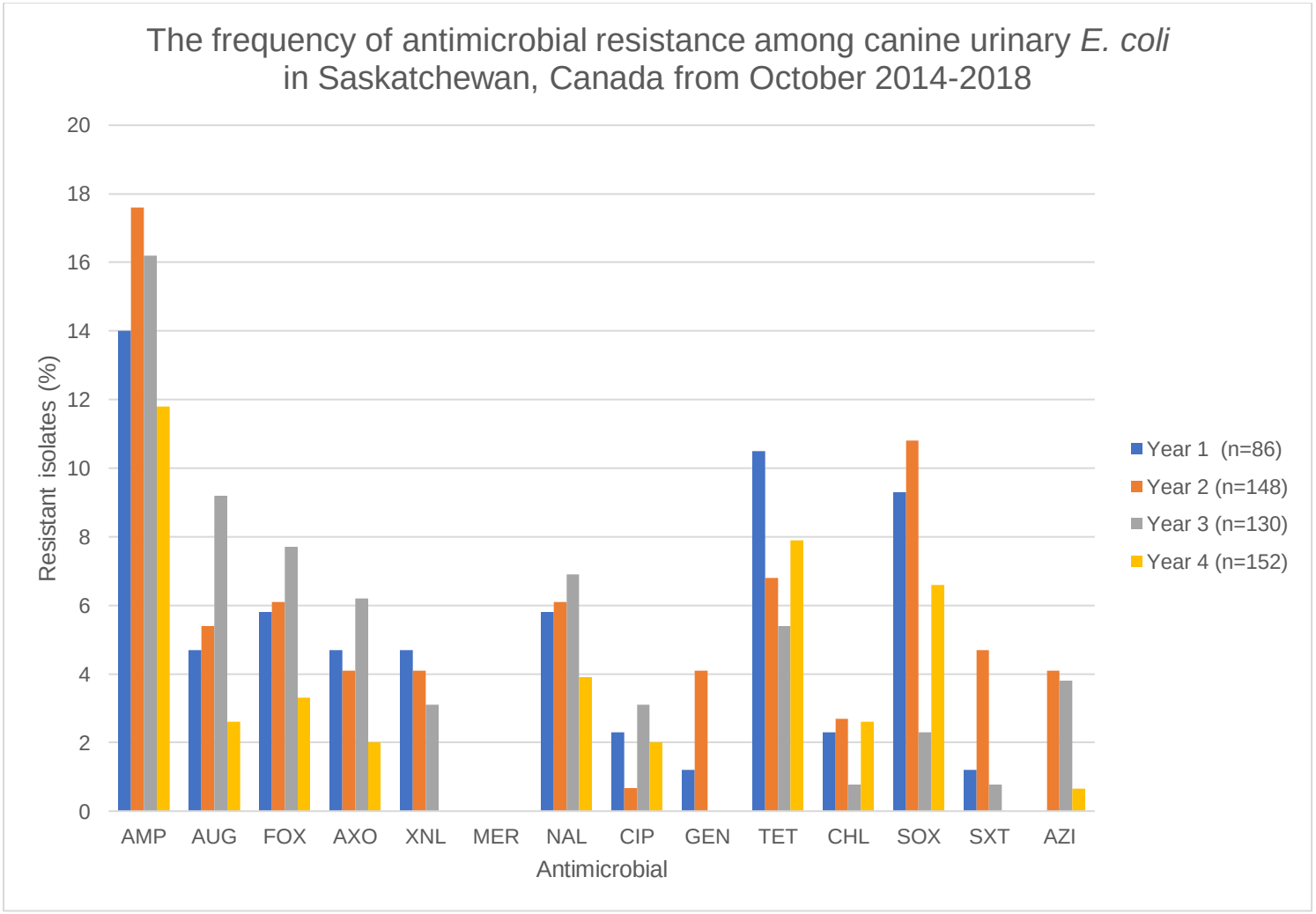

Figure 2.1: Number of isolates (n=516) exhibiting resistance across four years of a canine urinary *E. coli* antimicrobial resistance surveillance program. AMP- ampicillin, AUG- amoxicillin-clavulanate, FOX- cefoxitin, AXO- ceftriaxone, XNL- ceftiofur, MER- meropenem, NAL- nalidixic acid, CIP- ciprofloxacin, GEN- gentamicin, TET- tetracycline, CHL- chloramphenicol SOX- sulfisoxazole, SXT- trimethoprim-sulfamethoxazole, and AZI- azithromycin

	Percentage of all isolates with MIC (μg/mL)																
Antimicrobial	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16.0**	**32.0**	**64.0**	**128**	**256**	**512**	**Resistant (%)**
Ampicillin							1.9	22.7	50.8	8.7	0.97	0	14.9				14.9
Amox/Clav							0.78	5.2	57.9	27.9	2.7	2.1	3.3				5.4
Cefoxitin						0	1.2	9.3	48.4	30.6	4.8	2.3	3.3				5.6
Ceftriaxone					94.2	0.97	0.39	0.39	0.19	0.19	1.4	0.58	0	1.7			4.1
Ceftiofur[1]				3.0	12.3	65.9	12.3	1.3	0.66	1.3	3.3						4.6
Meropenem[1]			99.1	0.94	0	0	0	0	0	0							0
Nalidixic Acid						0.39	4.7	53.1	33.5	2.5	0.19	0	5.6				5.6
Ciprofloxacin	53.3	36.8	2.7	2.1	1.6	1.2	0.39	0	0	1.9							1.9
Gentamicin					3.1	41.3	46.9	5.6	1.6	0.19	0.19	1.2					1.4
Tetracycline									89.9	2.7	0.78	0.39	6.2				7.4
Chloramphenicol								0.58	4.3	59.3	33.7	0.97	1.2				2.1
Sulfisoxazole											51	30.2	9.1	1.9	0.58	7.2	7.2
SXT				95.9	1.4	0.58	0.39	0	0	1.7							1.7
n=516																	

[1]N=302 for ceftiofur as ceftiofur was replaced with meropenem in the most recent version of the NARMS panel (CMV4AGNF) released in 2017

Figure 2.2: An MIC distribution of canine urinary *E. coli* isolates (n=516) collected from November 2014-October 2018. Breakpoints (indicated in red) are based off of published CLSI standards

Table 2.5: Resistance profiles of multidrug resistant isolates (n=31)

Phenotype	Number of Isolates
AMP + TET + SOX	5
FOX + NAL + CHL	1
TET + SOX + AZI	1
AMP + TET + SOX + SXT	1
AMP + NAL + CIP + SOX	1
AMP + NAL + SOX + SXT + AZI	1
AMP + GEN + SOX + SXT + AZI	1
AMP + AUG + FOX + TET + SOX	1
AMP + AXO + NAL + CIP + SOX	1
AMP + AXO + TET + CHL + SOX	1
AMP + FOX + TET + CHL + SOX	1
NAL + CIP + TET + CHL + SOX	1
AMP + AUG + FOX + AXO + TET + SOX	1
AMP + TET + CHL + SOX + SXT + AZI	1
AMP + AUG + FOX + AXO + NAL + TET	1
AMP + NAL + CIP + TET + SOX + AZI	1
AMP + NAL + TET + GEN + SOX + SXT + AZI	1
AMP + AUG + FOX + XNL + NAL + GEN + SOX	1
AMP + AXO + XNL + NAL + TET + SOX + SXT	1
AMP + AXO + XNL + NAL + CIP + TET + CHL + AZI	1
AMP + AUG + FOX + AXO + XNL + TET + CHL + SOX	1
AMP + AUG + FOX + AXO + XNL + GEN + TET + SOX	1
AMP + AXO + NAL + CIP + TET + SOX + SXT + AZI	1
AMP + AUG + FOX + AXO + XNL + NAL + CIP + TET + SOX	2
AMP + AUG + FOX + AXO + XNL + GEN + TET + CHL + SOX	1
AMP + AXO + XNL + NAL + GEN + TET + CHL + SOX + SXT + AZI	1

2.4.3 Detection of resistance genes

Of 516 isolates, 4.3% (n=22) exhibited resistance to the third generation cephalosporins ceftriaxone (MIC≥4) and/or ceftiofur (MIC≥8). Of the ESBLs detected, CTX-M-27 was the most prevalent (n=3) followed by CTX-M-15 (n=2) and CTX-M-14 (n=2). One of the CTX-M 27 producers displayed resistance to all seven antimicrobial classes tested (Table 2.6). There was no significant change in the frequency of CTX-M producing isolates observed over the study period. SHV and TEM type ESBLs were not detected. Of 22 third generation cephalosporin resistant isolates, 68% were also cefoxitin resistant (MIC≥32), of which 12 produced CMY-2 type AmpC β-lactamases (Table 2.6). There was no significant change in the frequency of CMY-2 producing isolates across the study period. The overall occurrence of AmpC β-lactamases and ESBLs in our isolates collection was 2.3% and 1.3% respectively. Broad spectrum β-lactamase production (CTX-M or CMY-2 type enzymes) was significantly associated with MDR (p=0.0001) and fluoroquinolone resistance (p=0.0002). The PMQR determinant *aac(6')- Ib-cr* was identified in a single isolate that was also MDR to four antimicrobial classes. A broad spectrum β-lactamase (SHV, TEM, CTX-M or CMY-2) was not detected in this isolate.

Table 2.6: Phenotypic and genomic characteristics of β-lactamase producing *E. coli* isolated from canine urinary samples

Isolate	Year	Phenotype	Genotype
ECPDS140	1	AMP + AUG + FOX + AXO + XNL + GEN + TET + CHL + SOX	*bla*$_{CMY-2}$
ECPDS155	1	AMP + AUG + FOX + AXO + XNL + TET + CHL + SOX	*bla*$_{CMY-2}$
ECPDS202	1	AMP + AXO + XNL + NAL + TET + SOX + SXT	*bla*$_{CTX-M-15}$
[1]ECPDS218	2	AMP + AUG + FOX + AXO + XNL + NAL + CIP + TET + SOX	*aac(6')- Ib-cr*
ECPDS236	2	AMP + AXO + XNL	*bla*$_{CTX-M-15}$
ECPDS264	2	AMP + AUG + FOX + AXO + TET + SOX	*bla*$_{CMY-2}$
ECPDS272	2	AMP + AUG + FOX + AXO + XNL + GEN + TET + SOX	*bla*$_{CMY-2}$
ECPDS289	2	AMP + AUG + FOX + AXO + XNL + NAL + CIP + TET + SOX	*bla*$_{CMY-2}$
ECPDS306	2	AMP + AUG + FOX + XNL + NAL + GEN + SOX	*bla*$_{CMY-2}$
ECPDS318	2	AMP + AXO + XNL + NAL + GEN + TET + CHL + SOX + SXT + AZI	*bla*$_{CTX-M-27}$
ECPDS426	2	AMP + AUG + FOX + AXO + XNL	*bla*$_{CMY-2}$
ECPDS462	3	AMP + AUG + FOX + AXO + XNL	*bla*$_{CMY-2}$
[1]ECPDS464	3	AMP + AUG + FOX + AXO + XNL + AZI	-
[1]ECPDS465	3	AMP + AUG + FOX + AXO + XNL	-
ECPDS494	3	AMP + AXO + XNL + NAL + CIP + TET + CHL + AZI	*bla*$_{CTX-M-14}$
ECPDS537	3	AMP + AUG + FOX + AXO + NAL + TET	*bla*$_{CMY-2}$
ECPDS538	3	AMP + AUG + FOX + AXO + AZI	*bla*$_{CMY-2}$
ECPDS552	3	AMP + AXO + NAL + CIP + TET + SOX + SXT + AZI	*bla*$_{CTX-M-27}$
ECPDS580	3	AMP + AUG + FOX + AXO + NAL	*bla*$_{CMY-2}$
ECPDS682	4	AMP + AUG + FOX + AXO	*bla*$_{CMY-2}$
ECPDS698	4	AMP + AXO + NAL + CIP + SOX	*bla*$_{CTX-M-27}$
ECPDS749	4	AMP + AXO + TET + CHL + SOX	*bla*$_{CTX-M-14}$

[1]These isolates did not possess one of the β-lactamases that was screened for (SHV, TEM, CTX-M or CMY-2)

2.4.4 Molecular epidemiology

MLST was performed on seven ESBL producers, as well as the single isolate harbouring the *aac(6')- Ib-cr* gene (Table 2.7). Two isolates belonged to ST38; one possessed an *aac(6')- Ib-cr* gene, while the other produced the ESBL CTX-M-27. The remaining isolates belonged to distinct sequence types including ST10, ST12, ST117, ST131, ST648 and ST1193. The isolates producing CTX-M-14 belonged to ST117 and ST648. The isolates harbouring CTX-M-15 belonged to ST10 and ST12. The two remaining CTX-M-27 producers belonged to ST131 and ST1193.

Table 2.7: Phenotypic and genomic characteristics of CTX-M and PMQR producing *E. coli* isolated from canine urinary samples

Isolate	Phenotype	Genotype	ST
ECPDS202	AMP + AXO + XNL + NAL + TET + SOX + SXT	*bla*$_{\text{CTX-M-15}}$	ST10
ECPDS218	AMP + AUG + FOX + AXO + XNL + NAL + CIP + TET + SOX	*aac(6')- Ib-cr*	ST38
ECPDS236	AMP + AXO + XNL	*bla*$_{\text{CTX-M-15}}$	ST12
ECPDS318	AMP + AXO + XNL + NAL + GEN + TET + CHL + SOX + SXT + AZI	*bla*$_{\text{CTX-M-27}}$	ST131
ECPDS494	AMP + AXO + XNL + NAL + CIP + TET + CHL + AZI	*bla*$_{\text{CTX-M-14}}$	ST648
ECPDS552	AMP + AXO + NAL + CIP + TET + SOX + SXT + AZI	*bla*$_{\text{CTX-M-27}}$	ST1193
ECPDS698	AMP + AXO + NAL + CIP + SOX	*bla*$_{\text{CTX-M-27}}$	ST38
ECPDS749	AMP + AXO + TET + CHL + SOX	*bla*$_{\text{CTX-M-14}}$	ST117

2.5 Discussion and Conclusion

The most recent treatment guidelines from ISCAID recommend amoxicillin or SXT and, if amoxicillin alone is not available, amoxicillin-clavulanate for the empirical treatment of uncomplicated cystitis in canine patients[54]. On the other hand, nitrofurantoin, fluoroquinolones, doxycycline, chloramphenicol, and fosfomycin are considered alternative treatments for MDR pathogens[54]. Our findings suggest that canine urinary *E. coli* in Saskatchewan remained remarkably susceptible to commonly prescribed antimicrobials. In fact, the proportion of isolates resistant to sulfisoxazole and SXT, decreased significantly during the study period. While this decrease in resistance to sulfisoxazole is not particularly clinically relevant since sulfonamides are not used in isolation in companion animals, SXT is considered a good first line option for treating UTIs and has historically been commonly prescribed.

Resistance to ampicillin was most common (~15%), while fluoroquinolone susceptibility remained remarkably high (~98%) in this isolate population (n=516). This is in contrast to AMR trends observed in urinary *E. coli* of human origin[68,150–152,154]. In fact, a lack of efficacy coupled with the alarming rates of resistance reported recently, have caused the IDSA to remove both amoxicillin and fluoroquinolones from their treatment guidelines for uncomplicated cystitis. Specifically, fluoroquinolones are to be reserved for important uses other than cystitis[64]. In general, clinical guidelines recommend that local resistance rates exceeding 10% indicate an emerging resistance problem and the need to adjust empirical treatment choices for antimicrobial therapy[50]. While the ampicillin resistance rate reported here (~15%) is concerning, ampicillin is not the aminopenicillin of choice for treating canine patients with cystitis. Amoxicillin, the

oral formulation used in practice, achieves extraordinarily high concentrations in the urine and bladder in vivo, which exceed the breakpoint used to interoperate in vitro susceptibility results[54]. This fact highlights the limitations of using indicator drugs and the importance of integrating the results of laboratory tests with clinical judgment and knowledge of pharmacology, when making decisions about prudent AMU.

Two previous studies have investigated phenotypic and genomic resistance among canine urinary *E. coli* in this region. A previous investigation, corresponding to the first year of passive surveillance completed by our group, found similar rates of susceptibility to those identified here, including a significant proportion of isolates exhibiting pan susceptibility to all antimicrobials tested (79.6%), reduced susceptibility to ampicillin (8.8%), and extremely low levels of quinolone resistance with just 7% of isolates exhibiting resistance to nalidixic acid and <2% displaying ciprofloxacin resistance[122]. A single CMY-2 type AmpC β-lactamase producers was identified and no ESBLs were detected[122]. Similar to our findings, a 2008 study reported no change in the number of antimicrobials to which non recurrent urinary *E. coli* were resistant from 2002-2007[205]. They also reported an increase in susceptibility (as measured by an increase in antimicrobial impact factor) to SXT[205]. However, this study differed from ours in many ways. While our group performed passive surveillance through quantitative susceptibility testing of isolates collected in real time, this investigation presented a retrospective analysis of laboratory susceptibility data and did not perform any susceptibility or molecular testing. The authors also assessed and reported changes in susceptibility and clinical efficacy based on antimicrobial impact factor, which takes into account the prevalence of a pathogen. This is in contrast to the current study, where we

report MICs and corresponding rates of susceptibility and resistance on an agent by agent basis over time. These factors make it somewhat difficult to draw comparisons between studies. However, from a surveillance perspective, the strict inclusion criteria and molecular techniques employed here provide a more complete picture of the state of resistance among urinary *E. col*i in this region.

From an epidemiological perspective our results have allowed us to describe the emergence of important AMR mechanisms in this region. In contrast to our previous findings, here we report the emergence of ESBLs of the CTX-M type, the PMQR determinant *aac(6')-Ib-cr* and the pandemic clone *E. coli* ST131 in companion animal urinary pathogens in Saskatchewan. CTX-M type ESBLs are a globally disseminated and highly transmissible form of ESC resistance that is most commonly reported among *E. coli* causing community acquired cystitis in humans[76]. CTX-M type enzymes have been identified in canine urinary *E. coli* in Australia, Europe, Asia and the U.S.[29,127,135,136,138,216,226,251,253–256,278]. In Canada, CTX-M-15 is the most common ESBL identified in the human literature[152,156,166] Though the frequency of CTX-M producers was low in our canine isolates, the alleles CTX-M-15 and -14 were detected in addition to CTX-M-27, which is closely related to CTX-M-14. Co-resistance to fluoroquinolones is a well-documented phenomenon among ESBL producing urinary *E. coli* in human medicine that was also found in the present study[166]. However, of the three fluoroquinolone resistant ESBL producers reported here none possessed the allele CTX-M-15, which is detected most frequently among isolates displaying fluoroquinolone resistance in the human literature[166].

The PMQR determinants *qnr*, *aac(6')-Ib-cr* and *qep*A have been particularly thoroughly investigated in the human literature. The *aac(6')-Ib-cr* gene is the most widely disseminated PMQR determinant, having been identified in *E. coli* from both healthy and diseased companion animals across Europe, Australia, Asia and the U.S.[135–139]. Here, we provide the first description of Aac(6′)-Ib-cr producing *E. coli* from companion animals in Canada. *E. coli* harboring *aac(6')- Ib-cr* have been detected often in community acquired UTIs from humans and frequently co-produce CTX-M type ESBLs[166,173]. The tendency of the *aac(6')- Ib-cr* gene to co-locate on plasmids harbouring ESBLs is of particular concern, as it provides the basis for the emergence of MDR strains. In fact, here we identify *E. coli* ST131 for the first time in a clinical isolates of companion animal origin in Canada. This globally prevalent clone is one such example where fluoroquinolone resistance and ESBL production came together to produce a highly pathogenic and MDR urinary *E. coli* strain[186].

Epidemiologically, AMR spreads in one of two ways; by the clonal dissemination of a single resistant strain or by non-clonal mechanisms including the horizontal transfer of resistance determinants in an otherwise heterogenous population. While the high prevalence of ST131 among community acquired UTIs in the human literature is one such example of clonal spread, the results of the current study more closely reflect the second mechanism[185,186]. Not only did the seven ESBL producing isolates identified exhibit seven distinct phenotypic resistance profiles, but the results of MLST indicate that these isolates also belonged to seven distinct lineages (Table 2.7). Taken together, these findings suggest that the population of urinary *E. coli* in our region is genetically heterogenous and that resistance has most likely emerged by the horizontal

dissemination of resistance genes on mobile genetic elements. In fact, among companion animal pathogens, there is a lack of evidence for a concrete linkage between a specific gene and a certain sequence type. For example, in *E. coli* isolates, ESBL production has been identified in a variety of sequence types[255,257,279,280]. However, there is some evidence that ESBL production is more frequently identified among *E. coli* belonging to certain lineages (ST10, ST23, ST38, ST88, ST131, ST167, ST410 and ST648) including those identified in this investigation (ST10, ST38, ST131 and ST648)[257,279,281,282].

The current study applied a rigorous inclusion criteria and gold standard microbiological methods, including quantitative susceptibility testing and molecular epidemiological techniques (MLST) to describe AMR among canine urinary *E. coli* in Saskatchewan. Our susceptibility testing methods were chosen over other agar-based techniques in an attempt to balance cost with the informative nature of the data obtained and the time required to perform the assay. Disk diffusion is a technique commonly employed in veterinary diagnostic labs. However, compared to the numeric MIC value obtained by microdilution methods, its results are limited to a qualitative description of bacterial phenotype as susceptible, intermediate or resistant[91]. This fact limits the information which may be generated from the results. Numerical MICs allows us to detect reduced susceptibility where bacteria are able to grow at increasing antimicrobial concentrations, while still remaining clinically susceptible. This phenomenon would be missed by disk diffusion methods because interpretation is strictly categorical. Such information is important as it provides clues regarding the molecular development of resistance. For example, while spontaneous mutations may

cause an increase in MIC by one or two dilutions, the acquisition of resistance genes by horizontal gene transfer tends to cause a substantial increase in MIC from susceptible into resistant ranges.

Despite these strengths, this investigation presents some limitations worthy of discussion. First, this was a passive surveillance study based on convenience sampling of laboratory isolates and as such our sample size is limited. Though every attempt was made to exclude repeat submissions, isolates submitted for culture and susceptibility in veterinary medicine are generally representative of treatment failures since most UTIs are initially treated empirically. It is also possible that isolates collected early on in the study period represent repeat submissions from before surveillance began. What's more, the vast majority of our isolates (~93%) originated from Saskatchewan, which significantly limits the generalizability of our results to the Western Canadian region we strove to capture. Second, there was a lack of clinical information regarding previous antimicrobial treatment, co morbidities, clinical outcome and AMU data that may provide a more complete explanation of risk factors for the emergence of resistance in canine *E. coli*. Finally, the low frequency of resistance detected here, coupled with the relatively short duration of this study, limits the statistical power of analyzing and drawing conclusions about changes in resistance over time. However, as the aim of this study was to identify and report trends and mechanisms of AMR in a surveillance fashion, many of these specific clinical questions are outside the scope of this investigation. Overall, there are very few limitations associated with susceptibility testing as even results that indicate a high frequency of susceptibility are very informative. However, the

lack of urine specific breakpoints for many drugs does limit our ability to say with complete confidence that an isolate is clinically resistant[9].

As the first of its kind in this region, the robust methodology applied here, provides a strong example of disease specific AMR surveillance and its application to companion animal pathogens. These findings offer invaluable information about region specific AMR trends for companion animal veterinarians in informing empirical treatment of UTI, while also establishing a baseline for future surveillance activities. What's more, the isolate collection acquired in this study may be drawn upon in future investigations to answer more specific clinical questions.

In conclusion, canine urinary *E. coli* remain largely susceptible to first line therapies, though resistance, particularly to the aminopenicillins, warrants continued monitoring. This is the first molecular description of ESBL and PMQR production in canine urinary *E. coli* in Canada, as well as the first report of *E. coli* ST131 from a companion animal.

2.6 Transition statement

In order to obtain an isolate collection that is reflective of canine UTIs caused by *E. coli,* 67 isolates were initially excluded. To further establish a population that is reflective of uncomplicated UTIs, 127 additional isolates were removed from analysis. These isolates represented cases where repeat urine samples were culture positive for *E. coli* from the same patient during the study period. In total, 643 canine urinary *E. coli* were collected across the four-year study. Therefore, repeat isolates represented a substantial proportion (~20%) of our collection. Though clinical data was both beyond the scope of this investigation and not readily available, these repeat submissions are suggestive of treatment failure despite clinicians having access to susceptibility results. While underlying pathology alone may be responsible for this apparent lack of antimicrobial efficacy, increasing frequency of MDR infections or a lack of availability of effective agents might also be contributing. In any case, there is a need to investigate alternative antimicrobials for the treatment of UTIs. One such agent that has been reached for in human medicine is fosfomycin. In fact, the most recent treatment guidelines from the IDSA (human) and ISCAID (veterinary) include fosfomycin. While in humans it is considered an appropriate empirical treatment for sporadic cystitis, in veterinary medicine, fosfomycin is reserved for infections where MDR has been demonstrated. Yet, very little is known about the in vitro susceptibility of canine urinary *E. coli* to fosfomycin. The following chapter aims to fill this knowledge gap by investigating fosfomycin resistance among *E. coli* and S. pseudintermedius isolated from canine UTIs in Saskatchewan.

3. FOSFOMYCIN RESISTANT *ESCHERICHIA COLI AND STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM CANINE URINARY TRACT INFECTIONS IN SASKATOON, SK

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Conceived of and designed experiments: RC, JER, MARP Sample preparation and performance of experiments: RC, MS, MARP Data analysis and primary authorship: RC

3.1 Abstract

Background: *E. coli* and *S. pseudintermedius* are the two most common causes of canine UTIs. The emergence of resistance to first line antimicrobials, including ESBL and AmpC β-lactamase producing *E. coli* and MRSP, pose a treatment challenge for veterinarians. Fosfomycin is an inhibitor of cell wall synthesis used in the treatment of uncomplicated UTIs in people, and rarely, companion animals. The objective of this study was to determine the frequency of fosfomycin resistance among canine urinary *E. coli* and *S. pseudintermedius* obtained from a local diagnostic laboratory.

Methodology: A total of 387 isolates, including *E. coli* (n=274) and *S. pseudintermedius* (n=113) from canine urine specimens were included. Laboratory requisitions were reviewed to ensure that only one isolate per patient was included. Fosfomycin MICs were determined by agar dilution. Plates containing serial dilutions of fosfomycin from 0.25-64 μg/mL + 25 μg/mL glucose-6-phosphate (G6P) were included, and MICs were interpreted utilizing EUCAST breakpoints for *E. coli* and *Staphylococcus* spp.

Results: The vast majority of isolates were susceptible to fosfomycin. Seven *E. coli* (2.6%) and four *S. pseudintermedius* (3.5%) were resistant. Of the fosfomycin resistant *E. coli*, one also possessed an CMY-2 type AmpC β-lactamase. Of the fosfomycin resistant *S. pseudintermedius*, three were methicillin resistant.

Conclusion: The increasing rate of AMR among the canine urinary pathogens *E. coli* and *S. pseudintermedius* mirrors the emergence of resistance seen in human infections. Our results indicate that fosfomycin resistance is not yet common in canine urinary pathogens, although continued surveillance is required to identify changes in resistance.

3.2 Introduction

The emergence of resistance to clinically important antimicrobials has veterinarians and physicians alike reaching for alternative treatments for common infections. Particularly troublesome, is the emergence of ESBL producing *E. coli*, as a cause of uncomplicated or sporadic cystitis, in both human and canine patients[69,73].

The phosphonic acid derivative fosfomycin, was originally discovered in 1969 and has long been approved in European and Asian countries for the treatment of UTIs[283]. It's unique structure, pharmacokinetics and mechanism of action make fosfomycin especially desirable for the treatment of cystitis. Fosfomycin inhibits cell wall synthesis in both Gram-negative and Gram-positive organisms by acting as a phosphoenolpyruvate (PEP) analogue and interfering with the enzyme UDP- GlcNAc enolpyruvyl transferase (MurA) involved in the committed step of peptidoglycan synthesis[284]. What's more, fosfomycin is bactericidal, excreted predominantly unchanged in the urine over a prolonged period and has very low toxicity[283]. Fosfomycin also boasts a broad spectrum of activity that includes infections caused by the Gram-positive cocci *S. aureus* (including methicillin resistant strains), *E. faecalis* (including vancomycin resistant strains), *S. epidermidis, E. faecium* and *S. pneumoniae*[283]. Fosfomycin tromethamine has only recently been approved in the United States for the treatment of uncomplicated cystitis caused by susceptible *E. coli* and *E. faecalis* strains. It is administered as a single oral dose containing 3 g of fosfomycin tromethamine[75]. This single dosing regime is attractive from the context of patient convenience and compliance, while simultaneously minimizing selective pressure for resistance as uropathogens are exposed for a relatively short period[77,283]. Despite its relative novelty

in North America, the most recent recommendations from the IDSA place fosfomycin in the category of appropriate empirical treatment options for uncomplicated UTIs, alongside nitrofurantoin, SXT and pivmecillinam[64].

Following its resurgence to clinical use in human medicine, mechanisms of resistance to fosfomycin have been extensively studied recently. Resistance to fosfomycin occurs by three main mechanism and may be either chromosomally or plasmid encoded in both Gram-positive and Gram-negative organisms[283]. Target modification, including chromosomal mutations in the active site of the MurA enzyme are encountered infrequently, though they have been described in resistant *E. coli*[285,286]. Acquired fosfomycin resistance due to impaired uptake occurs more frequently in clinical isolates. Specifically, mutations in the glycerol-3-phosphate (*glp*T) and hexose-phosphate (*uhp*T, *uhp*A) transporters or their regulatory genes is common[103,283]. Multiple fosfomycin-modifying enzymes; including a glutathione transferase (*fos*A), a thiol transferase (*fos*B) a hydrolase (*fos*X) and two kinases (*fom*A and *fom*B) have also been described in *E. coli*[76,103]. Many of the genes encoding these enzymes have been identified on plasmids in Gram-negative (namely *fos*A) and Gram-positive (*fos*B) organisms alongside additional resistance determinants[144,145]. In fact, the plasmid mediated resistance gene *fos*A3 has been identified in animals with no previous fosfomycin exposure, suggesting that resistance can be co-selected for in the face of exposure to other agents[103]. The first report of *fos*A3 in a companion animal occurred in dogs and cats in China in 2012 and 2013. Since then, *fos*A3 has emerged as the most common fosfomycin resistance mechanism among companion animals and appears to be particularly widespread in canine populations in China[146–148].

Though there are comparably few studies in North America, fosfomycin remains remarkable efficacious against urinary *E. coli* from both humans and companion animals, with susceptibility rates in excess of 95% among both ESBL and non ESBL producing populations[73,94,96,287]. Recently, the emergence of *fos*A3 has been documented in an *E. coli* isolate collected from a human patient with recurrent UTIs in Pennsylvania[288]. Compared to Gram-negative infections, even less is known about mechanisms of fosfomycin in Gram-positive organisms in North America. However, a 2014 study identified a particularly high frequency of canine MRSP harbouring *fos*B, even among phenotypically susceptible strains[98]. While all of these isolates originated from skin infections, as the second most common cause of canine cystitis, fosfomycin susceptibility in urinary *S. pseudintermedius* is worthy of investigation[289]. Given the lack of information regarding susceptibility of canine uropathogens to fosfomycin in Canada, this study aimed to describe fosfomycin susceptibility and mechanisms of resistance among *E. coli* and *S. pseudintermedius* isolated from canine UTIs in Saskatchewan.

3.3 Materials and Methods

3.3.1 Microbiological analysis and inclusion criteria

Canine diagnostic samples submitted to PDS in Saskatoon, Saskatchewan were collected from October 2013 to July 2016. Samples were obtained as pure cultures plated on 5% Columbia blood agar on a weekly basis. Initial bacterial identification was performed at PDS by biochemical testing (2013-2015) and MALDI-TOF mass spectroscopy (2015-2016). Biochemical tests included; for *E. coli*, metabolism of indole, TSI agar, urea and citrate; and for *S. pseudintermedius* the methyl red, coagulase, DNase, and hyaluronidase tests. All canine urinary *E. coli* and *S. pseudintermedius* submissions were initially included. In cases of suspicious colony morphology or contamination, bacterial identity as *E. coli* was confirmed based on lactose fermentation on MacConkey agar and the spot indole test[290,291]. Identification of *S. pseudintermedius* was confirmed based on colony morphology, Gram staining, coagulase, catalase and oxidase tests. Rarely, colony morphology and biochemical tests were insufficient for species identification. In these cases, PCR was employed to amplify a universal gene; *16S rRNA* or *cpn*60[260,261]. All isolates were saved for analysis in TSB + 15% glycerol at -80°C for analysis.

Inclusion criteria was applied such that only one submission per dog was included over the study period. Data extracted from the PDS database to confirm the identity of repeat samples include microbiological information, such as laboratory analytical procedure, bacterial species and specimen type; and patient information,

including animal name, species, breed, age, client surname and case number, where available.

3.3.2 Antimicrobial susceptibility testing

3.3.2.1 Preparation of media

Fosfomycin MICs were determined by the agar dilution method. Stock solutions of 500 μg/mL G6P solutions were made as 12 mL aliquots and saved at -80°C prior to use. All G6P solutions were passed through a sterile filter to prevent contamination. From a stock solution containing 2,560 μg/mL in water, nine 1:2 serial dilutions of fosfomycin were created. On the day of testing, an equivalent volume of 500 μg/mL G6P was added to each fosfomycin dilution.

For AST ten plates containing nine, two-fold dilutions of fosfomycin (0.25-64 μg/mL) were used, plus a negative control containing only G6P. To each 18 mL tube of Mueller Hinton agar, 2 mL of the appropriate fosfomycin/G6P combination were added (1 mL sterile water + 1 mL G6P for control plate) such that each plate contained 1mL fosfomycin + 1 mL 25 μg/mL G6P. Plates were poured, left to cool and stored at 4°C overnight. All media was created less than 24 hours prior to susceptibility testing.

3.3.2.2 Susceptibility testing (agar dilution)

Susceptibility testing was performed according to the agar dilution method. Briefly, standard 0.5 McFarland suspensions were created in sterile water for each organism (*E. coli* and *S. pseudintermedius*) using a fresh overnight culture plated on 5% Columbia agar. Each suspension was diluted 1:10 and 2 μLs of the subsequent solution

was inoculated onto each of the ten agar plates containing fosfomycin + 25 μg/mL G6P using a multichannel pipette. Each plate was inoculated with three quality control strains (*E. faecalis* ATCC 29212, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213), one negative control and twenty-eight test isolates based on a pre-determined schematic. Following incubation at 35°C for 18-24 hours growth was observed, and the MIC determined manually. Fosfomycin susceptibility was interoperated according to the breakpoints published by EUCAST. In the case of *E. coli*, these values were for urinary isolates specifically. However, as there are currently no official fosfomycin breakpoints published specifically for *S. pseudintermedius*, the criteria for *Staphylococcus* spp. was used (Table 3.1 and Table 3.2).

3.3.2.3 Susceptibility testing (broth microdilution)

Susceptibility testing to a panel of additional antimicrobials was performed using the broth microdilution technique as follows. Standard 0.5 McFarland suspensions were prepared for each organism, in commercially obtained sterile water (Thermo Fisher Scientific) using a fresh overnight culture plated on 5% Columbia agar. A 30 μL volume of the bacterial suspension was transferred to Sensititre Mueller-Hinton broth. Following the application of a dosing head, 50 μLs was auto inoculated onto 96 well plates containing two-fold serial dilutions of various antimicrobials (Table 3.1 and 3.2). Plates were sealed and following overnight incubation at 35 °C, growth was observed and recorded manually on plate schematics obtained on the manufacturer website. We employed the Gram-negative (CMV3AGNF) and Gram-positive (GPALL1F) panels for *E. coli* and *S. pseudintermedius* respectively. The specific antimicrobials and

concentrations included are summarized in Table 3.1 and 3.2. To ensure the AST system performed as expected, quality control strains, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were run in parallel with test isolates. Results were interoperated according to standardized criteria (breakpoints) published by CLSI in their M100 document[262]. These values have taken into account microbiological, pharmacologic and clinical data to synthesize the efficacy of certain drug-bug combinations for the infection under investigation and are summarized in Table 3.1 and 3.2. Since some antimicrobial agents tend to concentrate in the urine high above levels attainable in the blood, where available urine specific breakpoints were used[209]. In addition to resistance to these drugs, we also report rates of MDR which was defined as resistance to at least one antimicrobial in three or more classes[263]. For the purposes of this study, we consider all β-lactams as one class.

Table 3.1: Antimicrobials, concentrations and breakpoints used for susceptibility testing of *E. coli* (CMV3AGNF)

Antimicrobial	Concentrations (μg/mL)	CLSI breakpoint (μg/mL)
Cefoxitin (FOX)	0.5-32	≥32
Azithromycin (AZI)	0.12-16	≥32
Chloramphenicol (CHL)	2-32	≥32
Tetracycline (TET)	4-32	≥16
Ceftriaxone (AXO)	0.25-64	≥4
[1]Amoxicillin-clavulanate (AUG)	1/0.5-32/16	≥32/16
Ciprofloxacin (CIP)	0.015-4	≥4
Gentamicin (GEN)	0.25-16	≥16
Nalidixic Acid (NAL)	0.5-32	≥32
Ceftiofur (XNL)	0.12-8	≥8
Sulfisoxazole (FIS)	16-256	≥512
Trimethoprim-sulfamethoxazole (SXT)	0.12/2.38-4/76	≥4/76
Ampicillin (AMP)	1-32	≥32
[2,3]Fosfomycin (FOS)	0.25-64	≥64 (EUCAST)

[1]Amoxicillin-clavulanate contains amoxicillin and clavulanic acid in a 2:1 ratio

[2]Fosfomycin susceptibility testing was performed using the agar dilution technique

[3]Fosfomycin breakpoints were obtained from the European Committee on Antimicrobial Susceptibility testing.

Table 3.2: Antimicrobials, concentrations and breakpoints used for susceptibility testing of *S. pseudintermedius* (GPALL1F)

Antimicrobial	Concentrations (μg/mL)	CLSI breakpoint (μg/mL)
Chloramphenicol (CHL)	2-16	≥32
Daptomycin (DAP)	0.5-4	
Gentamicin (GEN)	2-16	≥16
Linezolid (LZD)	1-8	≥8
Rifampin (RIF)	0.5-4	≥4
Trimethoprim-sulfamethoxazole (SXT)	0.5/9.5-4/76	≥4
Quinupristin/dalfopristin (SYN)	0.5-4	≥4
Tetracycline (TET)	2-16	≥16
Tigecycline (TGC)	0.03-0.5	
Oxacillin + 2% NaCl (OXA)	0.25-4	≥4
Ampicillin (AMP)	0.12-8	
Penicillin (PEN)	0.06-8	≥0.25
Vancomycin (VAN)	0.25-32	≥16
Levofloxacin (LEVO)	0.25-4	≥4
Ciprofloxacin (CIP)	1-2	≥4
Moxifloxacin (MXF)	0.25-4	≥2
Erythromycin (ERY)	0.25-4	≥8
Clindamycin (CLI)	0.5-2	≥4
Streptomycin (STR)	1000	
Nitrofurantoin (NIT)	32-64	≥128
[1,2]Fosfomycin (FOS)	0.25-64	≥64 (EUCAST)

[1]Fosfomycin susceptibility testing was performed using the agar dilution technique

[2]Fosfomycin breakpoints were obtained from the European Committee on Antimicrobial Susceptibility testing. Breakpoints for *S. aureus* were used for *S. pseudintermedius*

3.3.3 Molecular investigation

DNA was crudely extracted for PCR by boiling. From fresh overnight culture, four to five bacterial colonies were transferred to 200 μL of sterile water and heated at 95°C for ten minutes. The resulting suspension was spun down and the supernatant removed and stored at -20°C prior to use. If resistance phenotypes involved third generation cephalosporins, cephamycins, fluoroquinolones or fosfomycin (*E. coli*) or oxacillin or fosfomycin (*S. pseudintermedius*), isolates were sequentially screened for various resistance genes by PCR. General PCR conditions included denaturation at 94°C for 6 minutes followed by 30-35 cycles of denaturation (94°C), annealing (various temperatures), and extension (72°C) for 1 minute each. All reactions included a final extension step for 10 minutes at 72°C. All PCR reactions included a no template negative control and where possible, a positive control containing DNA from an isolate previously determined to be positive for the gene of interest. Primers and associated annealing temperatures utilized for all reactions are shown in Table 3.3.

3.3.3.1 Fosfomycin inactivating enzymes

Isolates displaying MICs above the EUCAST epidemiological cut-off of ≥16 μg/mL (for *E. coli*) and ≥64 μg/mL (for *S. pseudintermedius*) were screened for transferable fosfomycin resistance genes by PCR. *E. coli* were screened for *fos*A, *fos*A3 and *fos*C2, while *S. pseudintermedius* were screened for *fos*B using specific primers outlined in Table 3.3.

3.3.3.2 *S. pseudintermedius*

Oxacillin resistant *S. pseudintermedius* isolates were screened for *mec*A using primers outlined in Table 3.3.

3.3.3.3 *E. coli*

All third-generation cephalosporin (ceftriaxone or ceftiofur) resistant *E. coli* isolates were initially screened using universal primers for CTX-M type enzymes. Any positive isolates were sequentially screened for CTX-M groups 1, 2, 8 and 9, using primers described previously. Isolates were additionally screened for the SHV and TEM type ESBLs. Cephamycin (cefoxitin) resistant isolates were screened for CMY-2 type AmpC β-lactamase production. (Fluoro)quinolone resistant *E. col*i isolates were screened for each of the PMQR determinants; *qnr*A, *qnr*B, *qnr*S, *aac(6')-Ib-cr*, and *qep*A. The primers and associated annealing temperatures used for all reactions are outlined in Table 3.3.

Table 3.3: Primers and PCR conditions

Name	Nucleotide sequence (5'-3')	Amplicon size	Annealing Temperature (°C)	Reference
16S (1476/1478)	GAGTTTGATCCTGGCTCAG	500	60°C	[261]
	G(TA)ATTACCGCGGC(TG)GCTG			
AAC(6')-IB-CR	CATTGTAGCACGTGTGTA	482	67°C	[264]
	CATTGTAGCATGCG'I"I'GAAG			
CMY-2	ATGATGAAAAAATCGTTATGCTGC	1000	68°C	[266]
	GCTTTTCAAGAATGCGCCAGG			
CPN60 (1594/1595)	CGCCAGGGTTTTCCCAGTCACGACGACGTCGCCGGTGACGGCACCACCAC	700	57°C	[260]
	AGCGGATAACAATTTCACACAGGACGACGGTCGCCGAAGCCCGGGGCCTT			
CTX-M G1	GTTGTTAATTCGTCTCTTCC	700	61°C	[267,268]
	AGTTTCCCCATTCCGTTTC			
CTX-M G2	ACTCAGAGCATTCGCCGCTCA	1000	58.3°C	[268]
	TTATTGCATCAGAAACCGTG			
CTX-M G8	CGCTTTGCCATGTGCAGCACC	307	64°C	[269]
	GCTCAGTACGATCGAGCC			
CTX-M G9	GACCGTATTGGGAGTTTGAG	600	48°C	[270,271]
	ATCTGATCCTTCAACTCAGC			
CTX-M U	ATGTGCAGYACCAGTAARGTKATGGC	593	61°C	[267]
	TGGGTRAARTARGTSACCAGAAYCAGCGG			
FOSA	ATCTGTGGGTCTGCCTGTCGT	271	59.5°C	[147]
	ATGCCCGCATAGGGCTTCT			
FOSA3	CCTGGCATTTTATCAGCAGT	234	58°C	[146]
	CGGTTATCTTTCCATACCTCAG			
FOSB	TTTTGAGCTTGCAGGCCTAT	420	60°C	*
	ATGACCATCAGGGTCGGTAA			
FOSC2	TGGAGGCTACTTGGATTTG	217	50.5°C	[147]
	AGGCTACCGCTATGGATTT			
MECA	TCCAGATTACAACTTCACCAGC	250	52°C	[292]
	CCACTTCATATCTTGTAACG			
	ACCAATGCTTAATCAGTGAG			
QEPA	GCAGGTCCAGCAGCGGGTAG	199	60°C	[272]
	CTTCCTGCCCGAGTATCGTG			
QNRA	ATTTCTCACGCCAGGATTTG	516	53.4°C	[273]
	GATCGGCAAAGGTTAGGTCA			

QNRB	GATCGTGAAAGCCAGAAAGG	469	67°C	
	ACGATGCCTGGTAGTTGTCC			
QNRS	ACGACATTCGTCAACTGCAA	417	53°C	
	TAAATTGGCACCCTGTAGGC			
SHV	TTATCTCCCTGTTAGCCACC	797	68°C	[274]
	GATTTGCTGATTTCGCTCGG			
TEM	GCGGAACCCCTATTTG	964	58.3°C	[275]
	ACCAATGCTTAATCAGTGAG			

*Primers designed using Primer3 based off of sequence from ARGANNOT accession number AHLO01000073:63139-63558:417 (2016)

3.3.3.4 PCR purification and sequencing

Following PCR reactions, amplicons were further purified using the EZ-10 Spin Column PCR Purification Kit, (Bio Basic Canada Inc) according to the manufacturer's instructions. DNA sequencing was performed by Macrogen Inc. Obtained sequences were compared to reference genes published in the NCBI and CARD databases using the BLAST algorithm.

3.3.3.5 Conjugation study

To identify plasmids bearing fosfomycin resistance genes conjugation studies were performed on all fosfomycin resistant *E. coli*, using an azide resistant strain (*E. coli* J53) as a recipient. In short, Muller-Hinton agar plates were prepared containing the following combinations of antimicrobial compounds; azide alone (100 μg/mL), fosfomycin at 16 μg/mL (high), fosfomycin at 4 μg/mL (low), fosfomycin high (16 μg/mL) + azide (100 μg/mL), and fosfomycin low (4 μg/mL) + azide (100 μg/mL). From a fresh overnight culture, one or two colonies were inoculated in 2 mL of TSB broth and incubated at 35°C for three hours. Following incubation, 800 µL of recipient broth (J53) was combined with 200 µL of donor broth (test isolate) in 2 mL of fresh TSB and incubated overnight at 35°C. For quality control, 10 μL of each isolate (test and *E. coli* J53) in broth was sub cultured onto each of the five antimicrobial containing media and incubated overnight at 35°C. Once quality control plates were confirmed to perform as expected, 200 µL of each conjugation mixture was sub-cultured onto Muller-Hinton agar containing azide (100 μg/mL) + fosfomycin (16 μg/mL) and azide (100 μg/mL) + fosfomycin (4 μg/mL) and incubated overnight at 35°C. Where positive growth occurred,

transconjugates were plated onto both MacConkey and blood agar. Following an additional night of incubation, transconjugates were save back in TSB + 15% glycerol at -80°C for later analysis.

3.3.4 Statistical Analysis

The Fisher's exact test ($p<0.01$) was used to identify associations between fosfomycin and resistance to other antimicrobial compounds, as well as to the microbiological parameters MDR and pan-susceptibility to all other antimicrobials besides fosfomycin.

3.4 Results

3.4.1 Microbiological analysis and inclusion criteria

From October 1st, 2013 to July 1st, 2016, 337 *E. coli* isolates and 245 *S. pseudintermedius* isolates were obtained from PDS as part of ongoing surveillance efforts by our group. Non-urine, non-canine, non-*E. coli* or non-*S. pseudintermedius* and repeat isolates were excluded leaving a total of 274 canine urinary *E. coli* and 113 *S. pseudintermedius* isolates.

3.4.2 Antimicrobial susceptibility testing

Susceptibility to fosfomycin exceeded 97% among the 387 isolates tested. Overall, 7 (2.6%) *E. coli* and 4 (3.5%) *S. pseudintermedius* isolates were resistant (Figure 3.1). One fosfomycin resistant *E. coli* was MDR. Three of the fosfomycin resistant isolates were pan-susceptible to all other antimicrobials tested (Table 3.4). A statistically significant association ($p<0.01$) was found between resistance to fosfomycin and resistance to cefoxitin as shown in Table 3.5.

Of the four fosfomycin resistant *S. pseudintermedius*, 75% were also methicillin resistant (Table 3.4). A statistically significant relationship ($p<0.01$) was found between resistance to fosfomycin and resistance to oxacillin, clindamycin, gentamicin, the fluoroquinolones (levofloxacin, moxifloxacin and ciprofloxacin) and MDR as shown in Table 3.6.

3.4.3 Detection and transferability of resistance genes

None of the fosfomycin resistance genes *fos*A, *fos*A3, *fos*C2 or *fos*B were detected in our resistant isolates. A single fosfomycin resistant *E. coli* was found to produce the CMY-2 type AmpC β-lactamase and all three fosfomycin resistant MRSP possessed the *mec*A gene as shown in Table 3.4. Transmission of fosfomycin resistance was not found to occur between our resistant isolates and recipient *E. coli* J53 as determined by conjugation studies.

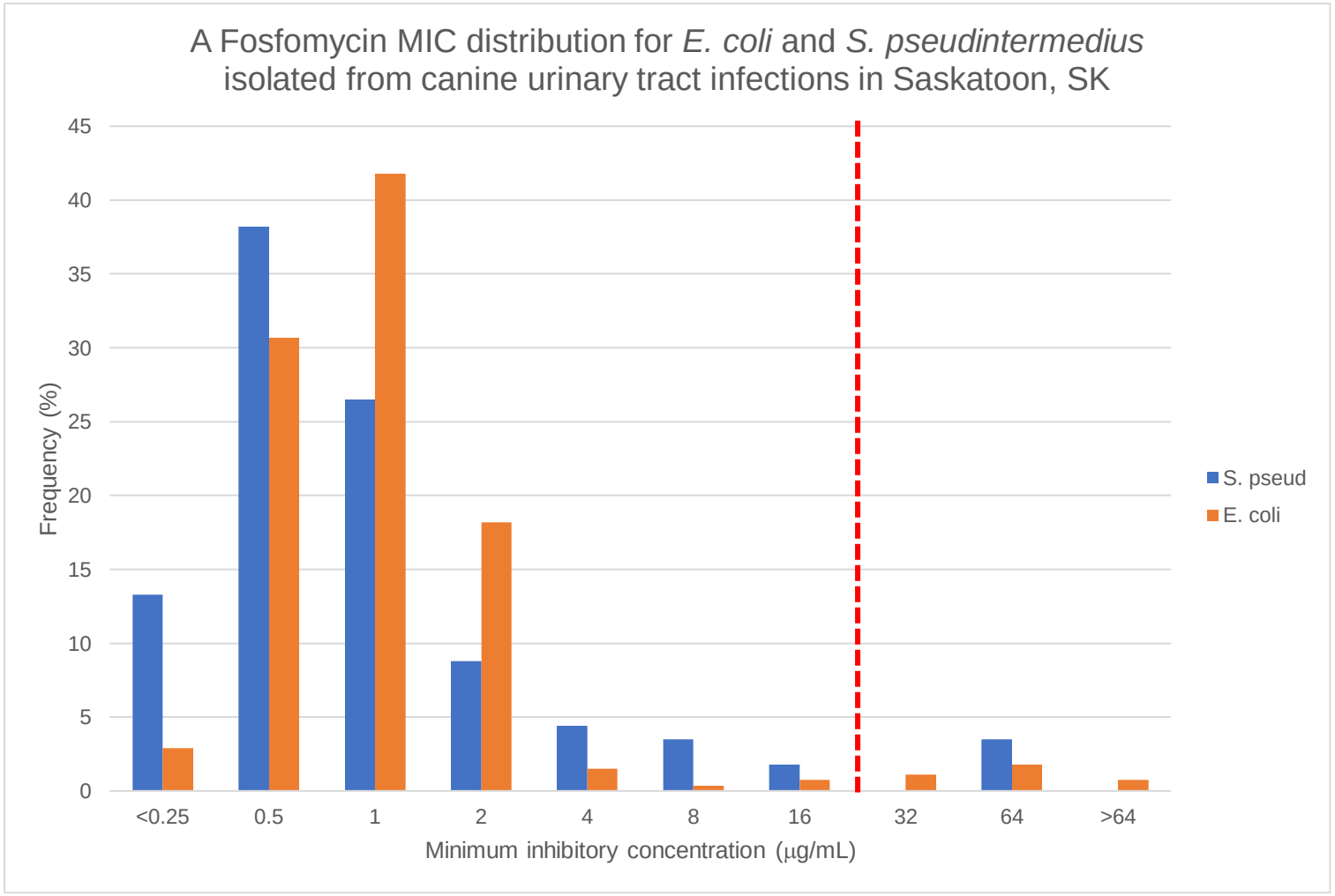

Figure 3.1: An fosfomycin MIC distribution of canine urinary *E. coli* (n=274) and *S. pseudintermedius* (n=113) isolates collected between October 2013-July 2016. Breakpoints (indicated by the red line) are based off of published EUCAST guidelines

Table 3.4: Phenotypic and genomic characteristics of fosfomycin resistant *E. coli* and *S. pseudintermedius* isolated from canine urinary samples

Isolate	Phenotype	Genotype
E. coli		
ECPDS22	FOS	
ECPDS84	FOS	
ECPDS109	FOS + AMP + AUG + FOX + AXO + XNL + TET	CMY-2
ECPDS114	FOS + AMP	
ECPDS177	FOS	
ECPDS249	FOS + FOX + NAL + CHL + SOX	
ECPDS304	FOS + AMP + AUG + AMP	
S. pseudintermedius		
SPPDS36	FOS + GEN + SXT + ERY + OXA + AMP + PEN+ LEVO + MXF + CLI + CIP	*mec*A
SPPDS81	FOS	
SPPDS87	FOS + SXT + ERY + OXA + AMP + PEN + LEVO + MXF + CLI + CIP	*mec*A
SPPDS130	FOS + GEN + RIF + SYN + OXA + AMP + PEN + CLI	*mec*A

Table 3.5: Fisher's exact test ($p<0.01$) for association between fosfomycin resistance and resistance to other antimicrobial classes among *E. coli* (n=274)

Antimicrobial	Number resistant (# co resistant to FOS)	p value
AMP	39 (3)	0.0622
AUG	12 (2)	0.0327
FOX	14 (3)	0.0033*
AXO	10 (1)	0.2314
XNL	10 (1)	0.2314
NAL	16 (1)	0.3469
CIP	3 (0)	1.00
GEN	6 (0)	1.00
TET	23 (1)	0.4625
CHL	9 (1)	0.2106
SOX	26 (0)	1.00
SXT	5 (0)	1.00
AZI	5 (0)	1.00
[1]Pan susceptible	214 (3)	0.0432
MDR	20 (1)	0.4153

[1]These isolates were susceptible to all antimicrobials besides fosfomycin.

Table 3.6: Fisher's exact test ($p<0.01$) for association between fosfomycin resistance and resistance to other antimicrobial classes among *S. pseudintermedius* (n=113)

Antimicrobial	Number resistant (# co resistant to FOS)	p value
PEN	68 (3)	1.00
AMP	44 (3)	0.2973
OXA	4 (3)	0.0001*
TET	25 (0)	0.5739
CHL	3 (0)	1.00
ERY	6 (2)	0.0135
CLI	4 (3)	0.0001*
GEN	4 (2)	0.0056*
LEVO	2 (2)	0.0009*
MXF	2 (2)	0.0009*
CIP	2 (2)	0.0009*
SXT	6 (2)	0.0135
RIF	1 (1)	0.0354
SYN	1 (1)	0.0354
[1]Pan susceptible	40 (1)	1.00
MDR	9 (3)	0.0014*

[1]These isolates were susceptible to all antimicrobials besides fosfomycin.

3.5 Discussion and Conclusion

As AMR continues to emerge in uropathogens, the importance of alternative therapeutic options such as fosfomycin increases in both human and veterinary medicine[69]. Currently, fosfomycin is recommended as an appropriate first line option for the treatment of uncomplicated cystitis caused by susceptible *E. coli* and *E. faecalis* in human medicine[64]. However, in the corresponding veterinary guidelines published by ISCAID, fosfomycin is reserved for the treatment of infections where MDR has been documented[54]. The results of the current investigation indicate that canine urinary *E. coli* in Saskatchewan are exceptionally susceptible to fosfomycin. Overall, these findings are consistent with resistance rates reported in other investigations. In human medicine, the microbiological and clinical literature summarizing the efficacy of fosfomycin against Enterobacteriaceae has been reviewed[78]. Of 13 microbiological studies, 10 looked exclusively at community or hospital acquired UTIs where the vast majority of isolates were ESBL producing *E. coli*. All ten studies reported susceptibility rates in excess of 90%[79–88]. Though the number of studies is limited in veterinary medicine, rates of susceptibility in excess of 97% are consistently reported[96,97,146,293]. Unfortunately, none of these investigations have looked exclusively at fosfomycin resistance among urinary *E. coli*. Fosfomycin is not labeled for use in companion animals and any veterinary use of this antimicrobial is therefore extra-label. In fact, the limited use of fosfomycin in veterinary medicine may be attributed to its formulation as a powder to be dissolved in water and taken orally once. Administration of this dose to canine patients therefore requires the use of a stomach tube that is generally not practical for uncomplicated cystitis cases in the face of more convenient oral formulations.

Similar to *E. coli*, our results indicate that canine urinary *S. pseudintermedius* are highly susceptible to fosfomycin. Though it is used much less commonly for the treatment of Gram-positive infections, in human medicine, methicillin susceptible and MRSA, penicillin resistant *S. pneumoniae* and vancomycin resistant *Enterococci* (VRE) have demonstrated high rates of susceptibility to fosfomycin[70]. In the present study we report a statistically significant relationship between methicillin and fosfomycin resistance. In fact, three out of the four MRSP isolates in our study were fosfomycin resistant and MRSP accounted for 75% (n=4) of fosfomycin resistant *S. pseudintermedius* isolates. This finding is supported by investigations into fosfomycin resistance among methicillin susceptible (MSSP) and resistant *S. pseudintermedius* causing canine pyoderma[98,99]. Specifically, while susceptibility among MSSP remained close to 100%, fosfomycin resistance among MRSP is closer to 15%[98,99]. However, given the low frequency of fosfomycin resistant *S. pseudintermedius* (n=4) identified in the present investigation, these results must be interoperated with caution.

In the present study, we did not identify any of the transmissible resistance genes *fos*A, *fos*A3, *fos*B or *fos*C2 among our isolates. What's more, though we did find a statistically significant relationship between fosfomycin and cefoxitin resistance, including one fosfomycin resistant *E. coli*, which coharbored a CMY-2 type AmpC β-lactamase, this relationship should be interoperated with caution due to small sample size. Interestingly, an association between CTX-M production and fosfomycin resistance has been previously reported among fosfomycin resistant *E. coli* of animal origin[144,145,147,294,295]. Considering the limited use of fosfomycin in companion animals, this affiliation is concerning as it supports the ability of *E. coli* to acquire incidental

fosfomycin resistance following treatment with common first line agents such as the β-lactams.

Despite the absence of inactivating enzymes, there are multiple alternative mechanisms, which may account for the fosfomycin resistance observed in this study[103]. In *E. coli,* decreased uptake and target modification by chromosomal mutations in the *mur*A gene have been described[76,103]. Specifically, mutations in the hexose phosphate (*uhp*A and *uhp*T genes) and glycerol 3 phosphate (*glp*T gene) transporter system involved in fosfomycin uptake, lead to decreased susceptibility or clinical resistance. These mechanisms may provide the basis for future molecular investigations of fosfomycin resistance within our isolate population. Interestingly, *fos*B has been reported to be highly prevalent (87%) among MRSP isolates, even in the absence of fosfomycin resistance[98]. However, we did not identify *fos*B among the *S. pseudintermedius* isolates investigated in this study. Additional studies are required to further elucidate this relationship in Staphylococci.

Limitations in the present investigation are mainly methodological and include first and foremost a limited sample size and relatively low frequencies of resistant isolates. There is also an overall lack of information regarding fosfomycin resistance in *S. pseudintermedius* in the literature. Specifically, the lack of an established species-specific breakpoint for *S. pseudintermedius* may have led to the inaccurate phenotypic categorization of isolates as resistant.

In conclusion, the increasing rate of AMR among the companion animal pathogens *E. coli* and S. *pseudintermedius* mirrors the emergence of resistance seen in

human infectious disease. Our results indicate that fosfomycin resistance, including transferrable resistance mechanisms, is not yet common in canine urinary pathogens. However, continued surveillance is required to identify changes.

4. GENERAL DISCUSSION AND CONCLUSION

Canine urinary pathogens in Saskatchewan remain susceptible to clinically relevant first line therapies, including the alternative agent fosfomycin. Except for ampicillin, resistance rates remain below 10% for all agents designated by ISCAID as either first line or alternative treatments for canine UTIs. Concerning resistance mechanisms that have become common place among human urinary *E. coli* have only recently been detected in this region, including ESBL producers, isolates possessing PMQR determinants, and members of the MDR pandemic lineage *E. coli* ST131. Resistance to the carbapenems, considered a last resort treatment for a variety of MDR Gram-negative infections in humans, was not detected. The significance of these findings resonates within the disciplines of public health, clinical veterinary medicine and epidemiology.

The public health threat posed by dogs (and other companion and production animals) in the zoonotic transmission of AMR genes has gained interest in recent years. Though strain sharing has been confirmed by molecular techniques in some instances, the direction of transmission remains poorly understood. In an attempt to quantify the contribution of animal sources to the human AMR problem, a sample of international microbiology experts were qualitatively surveyed[245]. Based on the results, among critically important human pathogens, the perceived involvement of animal reservoirs was estimated to be <4% and the majority of contributing diseases identified by specialists were well established zoonoses[245]. Various investigations have also highlighted significant discrepancies in the phenotypic and genomic resistance profiles of human and animal derived ExPEC. For example, the prevalence of ST131 is

particularly high among fluoroquinolone resistant *E. coli* of human origin (35%) compared to those from companion animals (7.2%)[296,297]. What's more, ESBL producing Enterobacteriaceae isolated in animals appear to be largely distinct form those encountered in humans[103]. These findings provide support for a relatively minor contribution of animal reservoirs to the AMR problem in human medicine, or, at the very least point to a more complex epidemiology of AMR emergence and dissemination in humans and animals.

On a global scale, the frequency of resistance in urinary *E. coli* in this region is comparable to results obtained in northern European countries including Denmark and Sweden[212,214]. The low frequency of resistance detected in this investigation is encouraging, as surveillance efforts so often represent a reactive as opposed to a proactive approach to the problem of AMR. Low levels of baseline resistance are informative for detecting resistance and classifying the molecular mechanisms responsible. As a natural phenomenon, the "novelty" of AMR is often measured only with respect to clinical relevance. In other words, resistance mechanisms that are believed to be newly described in clinical isolates are often not new at all and turn out to be prevalent once efforts to detect them are initiated. For example, the first case of mobile colistin resistance (mcr-1) from a commensal porcine *E. coli* isolate in China motivated an investigation into clinical Enterobacteriaceae isolates from hospital inpatients that revealed a low-level presence of mcr-1 (~1%)[298]. A susceptible population of clinical isolates like the one collected in this investigation, provides an excellent resource for retrospective investigations into these newly emerging resistance mechanisms.

There is significant pressure on veterinary professionals to use antimicrobials judiciously based on the perceived inappropriate use of these drugs in animals[15]. In the face of diagnostic uncertainty, pressure by owners and concern for secondary infections, local surveillance data and infection specific treatment guidelines provide support for clinicians in making empirical decisions regarding AMU[30]. In general, the “10% rule” is a useful starting point for interoperating surveillance data[50]. Where the frequency of resistance exceeds 10%, care must be taken in selecting an appropriate agent for empirical treatment. The case of amoxicillin resistance in human urinary *E. coli* is an excellent example of the value of surveillance data. Removed from the IDSA guidelines for UTI treatment in 1999 due to concern for resistance, twenty years later, this investigation identified an average ampicillin resistance frequency of 14% for canine urinary *E. coli* across the four-year surveillance study. While this figure exceeds the 10% rule generally used to guide interpretation of surveillance data, amoxicillin’s tendency to concentrate in the urine, coupled with the relatively low rates of resistance reported here, support its continued use in the empirical treatment of canine UTIs[209]. However, surveillance data is not a replacement for appropriate diagnostic work up or clinical judgment and must be interoperated within the clinical context. For example, the treatment of bacteriuria in the absence of clinical signs is considered unnecessary in the majority of cases in human medicine[299]. Similar care must be taken in selecting appropriate antimicrobials in recurrent or persistent UTIs, where underlying pathology complicates treatment[249]. In the end, decision making around AMU is complex in veterinary medicine and incorporates additional factors related to cost, commission, client convenience and availability, which may prevail even in the face of an emerging AMR problem.

4.1 Limitations

The limitations of this investigation can be broken into methodological and epidemiological. With respect to susceptibility testing, the broth microdilution and agar dilution methods used here, are both considered gold standard for the respective agents under investigation. However, it is possible that isolates displaying MICs which border on resistant may have been incorrectly classified based on the accuracy of the Sensititre AIM system used in this study. In order to get approved by the FDA, commercial devices must be accurate to within one double dilution, reporting false susceptible and false resistant results <1.5% and <3% of the time respectively[59]. Therefore, inaccurate MICs can be expected up to 1.5% or 3% of the time. An additional limitation associated with susceptibility testing is the interoperative criteria. Ideally, breakpoints take into account the pharmacokinetics of a specific drug formulation, at the specified label dose, in the veterinary species of interest, as well as clinical data on the likelihood of success in eradicating an infection in a particular body site[59]. Unfortunately, the published criteria for veterinary species, specifically dogs, is incomplete for many antimicrobials and is extrapolated from human data. For the purposes of surveillance and describing the frequency of in vitro resistance, we used the CLSI guidance document published for human infections throughout this study[262]. Therefore, it is possible that our interpretation of AMR is not reflective of clinical efficacy in this species. The only agent for which a urine specific breakpoint was used was for fosfomycin and *E. coli.* At the time of susceptibility testing, the only breakpoint available for fosfomycin (>32 μg/mL) was through EUCAST and this breakpoint differs from the one subsequently published by CLSI (≥ 256 μg/mL)[262]. What's more, there is currently no species-specific breakpoint for

fosfomycin against *S. pseudintermedius*, and thus we used the published value for the *Staphylococcus* spp. genus as a whole. To offset the potential error introduced by these breakpoints, we used the EUCAST epidemiological cut off values as a guide for our molecular investigation, to ensure that we captured all isolates with an MIC value above what would be expected in wild type *E. coli* or Staphylococci isolates.

The primary objective of this study was to identify and describe the frequency of AMR among urinary *E. coli*. However, a secondary aim included the molecular characterization of transferrable resistance to antimicrobials considered important for treating sporadic cystitis, namely β-lactams, fluoroquinolones and fosfomycin. Given the restricted nature of this part of the investigation there are many limitations to consider, which are largely outside the scope of a surveillance study. First, in cases were β-lactamases and PMQR determinants were not detected, it would have been interesting to pursue other resistance mechanisms including SNPs in the *par*C and *gyr*A genes encoding the antimicrobial target of fluoroquinolones and other broad spectrum β-lactamases such as additional CTX-M and other AmpC (CMY-1, FOX, ACT, MOX, ACC, DHA) type enzymes. To confirm transferability, it might have been beneficial to perform conjugation studies. However, given the surveillance nature of this study, as well as the substantial evidence for the localization of ESBLs and PMQR genes on plasmids, such studies were deemed unnecessary. Many additional fosfomycin resistance mechanisms have also been described in *E. coli* including decreased uptake and target modification by chromosomal mutations in the *mur*A gene[76,103]. Specifically, mutations in the hexose phosphate (*uhp*A and *uhp*T genes) and glycerol 3 phosphate (*glp*T gene) transporter systems could have been investigated. The DNA fingerprinting technique chosen in this

study, MLST, is also a potential limitation. While alternative “gold standard” strain typing methods, including WGS and PFGE may be considered superior, MLST allowed us to optimize cost and efficiency, while still providing adequate resolution[105-106]. Specifically, our exclusive interest in *E. coli* made MLST an appropriate technique, as it is able to discriminate between isolates of the same species. Unfortunately, MLST does not provide sufficient discrimination for all typing purposes, including resolving differences among single-clone organisms, as might be necessary in outbreak scenarios[101]. However, our interest in exploring the regional relatedness of MDR *E. coli* isolates to globally important strains, (ST131) makes this high level of resolution unnecessary.

A clearly defined objective is often overlooked in the planning and implementation of AMR surveillance initiatives. Specific goals can range from describing resistance in time and space, to identifying risk factors, to reporting the clinical or microbiological outcome of infections[149]. The inclusion criteria of the present investigation were simple and incorporated first time positive cultures for *E. coli* from canine urine samples over a four-year period. Previous AMU or other epidemiological information such as microbiological cure, clinical cure or underlying co morbidities were not considered. While this information would be considered if the goal was to ascertain risk factors for UTIs in canine patients, the objective, in this case, was strictly to identify and describe resistance in this region. However, additional sources of bias inherent in surveillance type studies must be considered, including case definitions, case ascertainment, sampling bias, multiple counting and laboratory procedures[149]. In the current investigation, case ascertainment and sampling bias are particularly important. As our data collection was based on convenience sampling of laboratory isolates, we failed to

collect certain samples fitting our case definition of uncomplicated canine UTIs caused by *E. coli*. What's more there is inherent sampling bias in passive surveillance studies of laboratory isolates, as this population is bias towards infections where empirical treatment has likely failed, leading to the submission of samples for culture and susceptibility. On the other hand, a combination of our strict inclusion criteria and robust methodology assisted in eliminating bias related to poor or incomplete case definitions, multiple counting of the same case, and laboratory procedures.

4.2 Future Directions

The development of this collection of canine urinary *E. coli* isolates allows for many microbiological and clinical questions to be investigated in the future. For example, this study does not address the interrelatedness between AMU and AMR. Acquiring and overlaying AMU data from medical records in future studies would allow for the association between previous antimicrobial exposure and the development of resistance to be investigated. What's more, with this clinical data, additional measures including clinical and microbiological efficacy may be established for various agents in treating uncomplicated cystitis in canine patients.

This investigation also did not consider how additional patient factors such as signalment, co-morbidities or underlying pathology might be contributing to the emergence of resistance. For example, exploring the resistance patterns among complicated and recurrent infections would be interesting to establish whether these infections are truly more MDR or if it is more likely a pathology or pharmacological

problem leading to treatment failure. It would also be interesting to perform a stratified analysis of certain populations including ESBL producers and ST131 isolates to try to establish the risk factors or resistance determinants associated with these infections.

Additional molecular investigations, including characterizing plasmids, confirming transmissibility by conjugation studies and obtaining more complete resistance genes profiles would also be informative. For example, efflux pump encoded by the *tet* and *flo*R genes confer resistance to tetracyclines and phenicols respectively, while the folic acid metabolic enzymes encoded by *sul* and *dfr* genes result in resistance to trimethoprim and sulfonamides[103]. Additional inactivating enzymes encoded by *tet*X and *cat* genes confer tetracycline and chloramphenicol resistance to *E. coli* respectively. Finally, there are additional SNPs in antimicrobial targets to consider, including the *rmt* and *arm*A genes that convey aminoglycoside resistance, as well as the *par*C and *gyr*A genes that lead to decreased fluoroquinolone susceptibility or resistance[103]. There are also many mechanisms left to explore regarding fosfomycin resistance in *E. coli*[103]. Characterizing SNPs in the hexose phosphate and glycerol 3 phosphate transporter systems encoded by *uhp*A, *uhp*T and *glp*T genes lead to decreased fosfomycin uptake and resistance, as does target modification by chromosomal mutations in the *mur*A gene[76,103]. PCR primers and conditions have been established previously for investigating these highly conserved genes in *E. coli*[286]. In fact, the extremely low number of fosfomycin resistant isolates identified (n=11) made WGS a viable option in this study. However, as the evidence for a connection between a SNP in one of these targets and phenotypic resistance is limited, the cost benefit ratio of performing WGS was considered unfeasible.

In human medicine there has been a lot of focus on the virulence factors and pathotypes of ExPEC[22,115,296,300,301]. Characterizing our isolates in this manner could answer questions related to the pathogenicity or virulence of canine urinary *E. coli,* as well as establish whether certain resistance genes are associated with certain pathotypes or virulence genes. Finally, as we know that resistance develops rapidly, but is often maintained and declines comparably slowly in a region or population, understanding the fitness costs associated with maintaining resistance genes and virulence factors is another interesting question to be pursued in the future.

4.3 Conclusion

Transmissible AMR mechanisms including ESBLs and PMQR genes are not yet common among canine urinary *E. coli* in Saskatchewan and these isolates remain highly susceptible to recommended empirical treatment options. Though it is considered a second line option by ISCAID, the alternative agent fosfomycin displays exemplary in vitro efficacy against both *E. coli* and *S. pseudintermedius* isolated from canine UTIs. Despite these optimistic results, consensus statements in veterinary medicine demand for more judicious use of antimicrobials, while the geographical variations in AMU and AMR require clinicians to be aware of emerging resistance in their practice area[15,49,50]. The findings generated by this research strives to fill this significant knowledge gap for practicing veterinarians and provide a baseline and blueprint for developing future AMR surveillance studies for companion animal pathogens in Canada.

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