Epidemiology of the virulence and antimicrobial resistance of *Escherichia coli* from canine and feline clinical isolates

by

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ABSTRACT

Escherichia coli resistance impacts the veterinary professional because of therapeutic failure and the public health significance. The lack of national monitoring programs in small animal practices limits evidence-based empirical antimicrobial choices. The objectives of the resistance studies were to establish the current status of *E. coli* susceptibility to routinely-selected antimicrobials and to assess veterinary prescribing behaviors.

Clinical canine or feline *E. coli* isolates (n=3172) were collected from six geographic regions in the US between May 2008 and January 2013. Minimum inhibitory concentrations (MIC) of *E. coli* isolates were determined for 17 antimicrobials. Population MIC distributions were bimodal with the second mode above the resistant breakpoint for all drugs except for gentamicin, amikacin, and meropenem. The highest percentage of isolates were resistant to doxycyline (100%), and cephalothin (98%). None of isolates was susceptible to all drug tested; 46% were single-drug resistant (SDR), and 52% multi-drug resistant (MDR).

Most antimicrobial prescriptions were amoxicillin-clavulanic acid (25%) at 14.9±5.4 mg/kg q12h and enrofloxacin (22%) at 5.6±2.8 mg/kg q24h used for treatment of urinary tract infections (UTI). Our findings reported that most antimicrobial prescriptions were inappropriate for the drug selected, and the dosing regimen.

Uropathogenic *E. coli* (UPEC) isolates acquire virulence genes that encode virulence factors (VF) necessary for colonization of the urinary tract. VFs may offer targets to alter a

method of antimicrobial therapy. The objectives of virulence studies were to investigate the associations among severity of UTI, antimicrobial resistance, and virulence within UPEC.

Virulence profiles of UPEC differ significantly with severity of infections and resistance patterns. Most severe disease isolates were phylogroups B2 (50%), and ABU were phylogroup D (63%). MDR isolates exhibited shifts in phylogenetic distribution (A, B1>D, B2), compared to SDR isolates (B2, D>B1, A). These results suggest that as the resistance level increases, virulence decreases.

The expression levels of virulence genes were computed by principal component and linear discriminant analyses to classify severity of UTI as ABU and non-ABU. The model estimated the non-ABU with a 19% error rate. These findings suggest that the model can be useful in practices by identifying those isolates that do not need antimicrobial therapy.

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LIST OF ABBREVIATIONS

AMC	Amoxicillin-clavulanic acid
AMK	Amikacin
AMP	Ampicillin
ATCC	American Type Cell Culture
CAZ	Ceftazidime
CEF	Cefazolin
CEP	Cephalothin
CHL	Chloramphenicol
CI	95% Confidence interval
CIP	Ciprofloxacin
CLP-AU	Clinical Pharmacology Laboratory at Auburn University
CLSI	Clinical Laboratory Standards Institute
CPD	Cefpodoxime
CTX	Cefotaxime
DOX	Doxycycline
ENR	Enrofloxacin
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum beta-lactamase
ExPEC	Extraintestinal pathogenic *E. coli*
FOX	Cefoxitin
GEN	Gentamicin

MDR	Multidrug resistant
MEM	Meropenam
MIC	Minimum inhibitory concentration
MIC_{BP}	CLSI MIC breakpoint
NC	North central
NDR	Non-drug resistant
NE	Northeast
NW	Northwest
OR	Odds ratio
PCR	Polymerase chain reaction
R%	Percent of resistance
R	Resistant
R-MIC_{BP}	CLSI MIC resistant breakpoint
S	Susceptible
SC	South central
SDR	Single drug resistant
SE	Southeast
S-MIC_{BP}	CLSI MIC susceptible breakpoint
SW	Southwest
SXT	Trimethoprim-sulfamethoxazole
TIM	Ticarcillin-clavulanic acid
UPEC	Uropathogen *E. coli*
UTI	Urinary tract infection

VF	Virulence factor
q12h	Every 12 hours
q24h	Every 24 hours
*q*RT-PCR	quantitative reverse transcription-PCR

CHAPTER 1

LITERATURE REVIEW

1.1 The evolution of pathogenic *Escherichia coli*

Escherichia coli is a genetically diverse bacterial species of considerable scientific, economic, and medical importance. The pathogenicity of *E. coli* ranges from nonpathogenic variant, strains with little ability to cause disease, and to pathogenic variants, i.e., enteropathogenic *E. coli* (EPEC) or extraintestinal pathogenic *E. coli* (ExPEC) in human and animal hosts [1]. The diversity within *E. coli* with pathogenic variants results from two fundamental evolution processes: mutation and horizontal gene transfer. Mutation involves the sporadic introduction of is a permanent change of the nucleotide sequence of various sizes throughout the genome [2] [3]. Mutation are transmitted vertically from generation to generation, resulting in a clonal population structure [4] [5]. Progressive diversification by mutation occurs in a dichotomously branching fashion through successive accumulation of mutations within lineages, with each new mutant derivative serving as the ancestor for a new branch of the phylogenetic tree [5]. Furthermore, horizontal gene transmission produces a web of genetic similarity relationships by creating cross-links between distinct branches of the clonally derived phylogenetic tree [6]. Therefore, the combination of mutation and horizontal transfer has shaped the overall phylogenetic structure of *E. coli.*

1.2 Virulence factors and virulence

Pathogenic *E. coli* variants derive their ability to cause intestinal and extraintestinal disease largely from their expression of multiple specialized virulence factors (VFs), including adhesins, toxins, siderophores, secretion systems, etc [7] [8] . These VFs are not required for simple commensalism, as evidenced by their absence from most commensal *E. coli* strains, e.g., the intestinal flora of healthy human and animal hosts. In contrast, the VFs provide the organism with an enhanced ability to colonize specific host surfaces, to avoid or subvert host defense systems, to stimulate a host inflammatory response, and to directly injure host cells and tissues, all of which can contribute to disease causation. Multiple functional categories of VFs and multiple representatives within each functional category typically are present in successful pathogenic *E. coli* strains, with the strain's net virulence potential varying in relation to the number and quality of VFs it possesses [1]. Thus, the evolution of pathogenic *E. coli* variants can be understood in terms of 1) the background evolution of the species as a whole (phylogeny), 2) the evolution of individual VFs, and 3) the interaction of the VFs with the genomic background, leading to focal concentrations of specific VFs within particular lineages of *E. coli*.

1.2.1 Phylogenetic distribution of VFs

Vertical transmission, the predominant mode of inheritance of VFs within *E. coli,* results in the linkage of particular VFs with specific phylogenetic lineages. VFs are passed from parent to progeny within these lineages, because of their constituent VFs [9] [10]. However, horizontal transmission has been appreciated increasingly as an important contributor to the creation of new virulent clones. VFs can be transferred horizontally between lineages by diverse genetic mechanisms and can move either as individual genes or as clusters of linked genes [11].

Different phylogenic groups are favored as the background development of intestinal versus extraintestinal pathogens, respectively, which are largely mutually exclusive pathotypic categories [8].

1.2.2 Phylogenetic analysis of E. coli

Initially, phylogenetic typing was based on cluster analysis of multilocus enzyme electrophoresis (MLEE) data, defined six major phylogenetic groups, designated A, B1, B2, C, D and E [12]. This phylogeny was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method [13], which makes the assumption of a strict constant rate of evolution across all lineages. Later, an analysis which is more robust methodology [14] was applied for 38 enzyme loci [15]. This analysis relaxes the assumption of evolutionary constant rate, and supports the category for the four major groups A, B1, B2 and D, with a few unclassified sequences that are sometimes referred to as group E (Fig. 1.1). Group A, which included *E. coli* K-12, is descendant of the phylogenetic tree, and is the earliest to diverge, with groups B1 and B2 as sister-taxa. Group C, which was defined in the earlier analysis as a paraphyletic group of divergent sequences at the base of group B, was not identified in this study and is no longer used.

The analysis of MLEE was used to identify the major phylogenetic groups in numerous studies by using a variety of data types which included restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) [16] , variation at mononucleotide repeat loci [17] and nucleotide sequence data [18]. A rapid classification of *E. coli* strains has been developed by Clermont et al. [19]. This study was using a triplex PCR to identify the four main phylogenic groups A, B1, B2, or D, by detecting the presence or absence of the genes *chuA*

(present in group B2 and D strains, absent from B1 and A) together with *yjaA* (present in group B2, absent from group D) or the DNA fragment TSPE4.C2 (present in group B1 strains, absent from group A) [19]. Phylogenetic analyses based on nucleotide sequence demonstrated that the analysis of triplex PCR allow typing 85-90% of *E. coli* strains, with 80-85% the correct phylogenetic groups [20] [21]. Since most analyses of MLEE are time-consuming and laborious, these methods are impractical to assess in an epidemiological study. Therefore, our study used the rapid triplex PCR to identify the phylotype of *E. coli* strains.

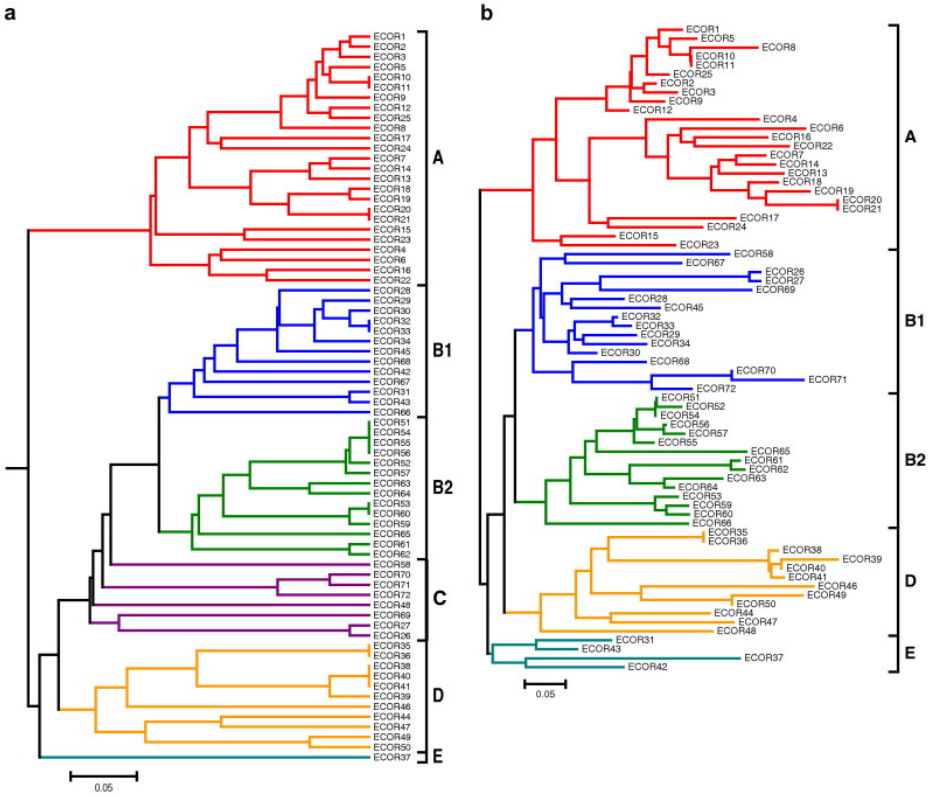

Figure 1.1 Phylogenetic relationships among the 72 *E. coli* reference strains, as assessed using multi-locus enzyme electrophoresis (MLEE). The groupings defined by the original authors are indicated. (a) Phylogeny constructed using the UPGMA method [13] with MLEE data from 35 enzyme loci, (b) Phylogeny constructed using the neighbour-joining method [14] with data from 38 enzyme loci [22] .

1.2.3 E. coli pathotypes

The evolutionary process has led to the emergence of the various pathogenic *E. coli* variants. Certain pathogenic strains cause enteric diseases ranging in symptoms from cholera-like diarrhea to severe dysentery; other *E. coli* may colonize the urinary tract, resulting in cystitis or pyelonephritis, or may cause other extraintestinal infections, such as septicemia and meningitis. There are at least eight recognized pathotypes of *E. coli* but many more distinct pathogenic clones. Figure 1.2 represents an attempt to illustrate the complex relationships among different pathotypes. It is useful to visualize pathogenic strains as belonging to two groups: those which cause gastrointestinal illness and those which cause extratintestinal infections. However, there may be strains with virulence potential that bridges these boundaries. Among the extraintestinal strains, it seems likely that most, if not all, strains capable of causing neonatal meningitis also can cause urinary tract infections (UTIs), although the converse may be very rare [23].

Diarrheagenic *E. coli* are classified into seven pathotypes including 1) enteropathogenic *E. coli* (EPEC), 2) enterotoxigenic *E. coli* (ETEC), 3) enteroaggregative *E. coli* (EAEC), 4) enteroinvasive *E. coli* (EIEC), 5) diffusely adherent *E. coli* (DAEC), 6) attaching-effacing (AAEC) and 7) shiga toxin-producing *E. coli* (STEC). STEC may also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). Extraintestinal pathogenic *E. coli* (ExPEC) strains include meningitis associated *E. coli* (MAEC) and uropathogenic *E. coli* (UPEC) [23].

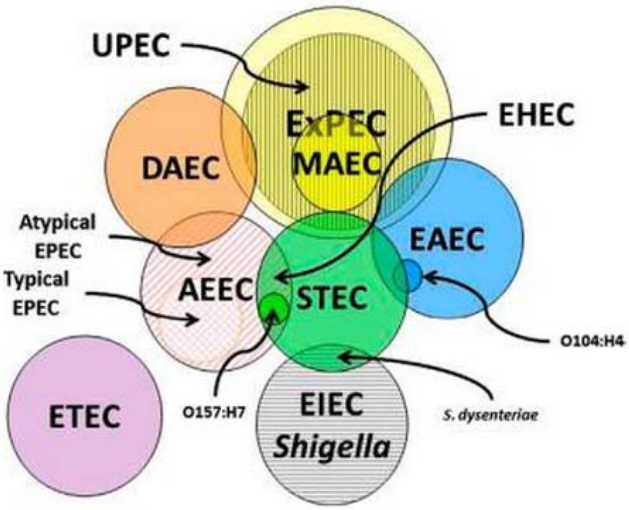

Figure 1.2 The complex relationship among different pathotypes of *E. coli* that cause disease in human as illustrated in a Venn diagram. Extraintestinal pathogenic *E. coli* (ExPEC) strains include meningitis-associated *E. coli* (MAEC), uropathogenic *E. coli* (UPEC), and strains from patients with pneumonia, cholecytitis, peritonitis, and other infections [23].

1.2.4 Phylogenetic distribution of pathogenic E. coli

Diarrheagenic *E. coli* is present in multiple phylogenetically distant lineages that might indicate that each pathotype of diarrheagenic *E. coli* has emerged independently multiple times within *E. coli* by horizontal acquisition of virulence genes, with convergent evolution yielding similar phenotypes despite the underlying phylogenetic diversity. The various diarrheagenic pathotypes occur almost exclusively in *E. coli* phylogenetic groups A, B1, and D and among the ungrouped strains, with the inferred distribution varying somewhat depending on the phylogenetic typing method used. They rarely derive from phylogenetic group B2. This is almost precisely the reverse of the phylogenetic distribution of ExPEC [1].

Most ExPEC, including those with the most robust VF repertories and those which are best able to infect noncompromised hosts, are derived from phylogenetic group B2. Most

currently recognized extraintestinal VFs are concentrated in or confined to group B2. Group D is the second largest contributor of ExPEC. Extraintestinal isolates from group D typically have both somewhat fewer VFs and a different mix of VFs than group B2 isolates [1].

In contrast to group B2 and D strains, strains from A and B1 do not commonly cause extraintestinal infection. Those which do usually either represent the exceptional members of these groups that by horizontal transfer have acquired sufficient extraintestinal VFs to cause disease in noncompromised hosts or are intrinsically low-virulence strains whose ability to cause invasive disease is largely limited to compromised hosts [1].

1.2.5 Pathophysiology of urinary tract infections (UTIs) by E. coli

E. coli is the most common pathogen that implicated in UTIs in dogs, cats, and humans. The infection is most frequently manifested as cystitis but urethritis, and pyelonephritis are also seen [24]. Thus, the review of this study will focus on the UTIs caused by *E. coli*.

Cystitis due to UPEC results from invasion of the bladder by bacteria present in the rectum, multiplication of the bacteria in the bladder, the inflammatory response to the UPEC, and tissue damage inflicted by the UPEC (Figure 1.3). The major steps in pathogenesis involve adherence of UPEC to the bladder epithelium, colonization, avoidance of host defense, and damage to the host tissue [25]. Urovirulent *E. coli* from feces initially colonize the periurethral area then move up into the urethra. Continued migration takes the UPEC into the bladder, where they adhere to bladder epithelial cells. Nevertheless, some UPEC are phagocytized and killed by neutrophils and some ExPEC are able to survive in neutrophil. Invasion of epithelial cells provides significant protection and longer term survival. Colonization requires recovery of iron from host, and this is done through a variety of siderophores. Damage to bladder epithelium is

largely the result of the inflammatory response including the toxic products of the neutrophils, but toxins produced by UPEC contribute as well. Infection does not often ascend to the kidneys [26].

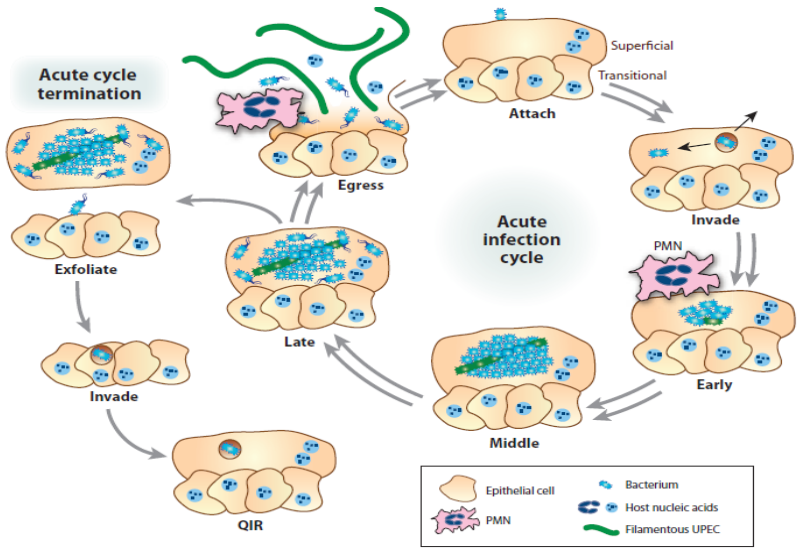

Figure 1.3 Schematic pathophysiology of urinary tract infection (UTI) associated with UPEC, with example virulence factors identified. Pathogenesis begins with bacterial adhesion (*pap, fim*) to uroepithelial cells. Toxins (e.g, cytotoxic necrotizing factor [*cnf*], hemolysins [*hyl*]) cause cellular damage to outer cells, facilitating penetration and providing nutrients for the organisms. Iron scavenging necessary for survival is mediated via siderophores (*ireA, iroN*). Additional adhesins such as Type 1 fimbrae (*fimAFGH*), FimH, allow recognition of mannose receptors of the uroepithelial cells while papillae (*pap*), genes allow renal colonization. FimH-mediated interactions with uroepithelium stimulate exfoliation of superficial cells, causing subsequent bacteriuria. Other adhesins (*papG*, *sfaS, foc*) signal structural changes in host cells and, along with formation of biofilm, infection transitions from an acute response to long-term persistence in the bladder wall [27]. QIR: quiescent intracellular reservoir; PMN: polymorphonuclear leukocyte.

E. coli that cause UTI typically originate from the dog's own intestinal tract and are characterized by a possession of a cluster of virulence genes [28]. Various UPEC may possess different combinations of VFs. The VFs play important roles as adhesins that mediate adherence to urinary tract epithelium, iron-scavenging systems that enhance survival in low-iron environments, and cytotoxic proteins that damage tissues. Pathogenesis involves a timely and stepwise expression of these VFs in hosts with formidable defense mechanisms which include the mechanical barrier imposed by the epithelium, flushing by frequent voiding of urine, and the inflammatory response [26].

1.2.6 Virulence factors of uropathogenic E. coli

1) Adhesins

Adherence plays a prominent role at the first step of colonization. *E. coli* express many different surface structures by which they bind to targets on the host cell surface. Fimbriae are common elements used by bacteria to adhere. Fimbriae distributed over the entire surface of the bacteria, giving the impression that the bacteria have a fringe of threads or hair covering their exterior [29].

UPEC strains have been shown to express a number of different fimbriae, including P, F1C, S, M, Dr, and type 1 fimbriae [30]. The fimbriae bind different host receptors and subsequently are believed to target different areas within the urinary tract [30]. Fimbriae were subdivided into two different groups based on a phenotypic assay for receptor specificity and categorized by their ability to hemagglutinate erythrocytes isolated from a variety of sources, including humans, sheep, and guinea pigs [31]. The ability of some fimbrial types to hemagglutinate erythrocytes was blocked by the presence of mannose, whereas other fimbriae

remained capable of hemagglutination in the presence of mannose. Therefore, fimbriae are categorized as mannose-sensitive (MSHA) or mannose-resistant (MRHA) based on the ability to hemagglutination erythrocyctes [31]. P fimbriae, S fimbriae, F1C fimbriae, and Dr fimbriae belong to MRHA, whereas type 1 fimbriae do not hemagglutinate erythrocytes in the presence of mannose, defined as MSHA [30].

1.1) Type 1

Type 1 fimbriae are mannose-binding hairlike projections expressed on the surface of *E. coli* and other members of the *Enterobacteriaceae*. The fimbriae function as a VF in the pathogenesis of *E. coli* UTI. Cystitis and pyelonephritis isolates have been observed to differ in the control of the invertible element orientation and the expression of type 1 fimbriae during the course of an infection [32]. Several studies have focused on the role of type 1 fimbriae in the invasion of host cells and host immune responses, and the development of a vaccine directed against type 1 fimbriae in UTIs [33].

Type 1 fimbriae are encoded by an operon consisting of nine separates genes present on the chromosome in the following order: *fimB*, *fimE, fimA, fimI, fimC, fimD, fimF, fimG* and *fimH* [34] [35]. The operon transcribes as at least three separate transcripts, one for encoding the main structure subunit of the fimbriae (*fimA*) and two others responsible for the adhesion (*fimH*) and accessory proteins necessary for assembly of the fimbriae [36].

The type 1 fimbriae bind, receptors that are widely distributed on epithelial surfaces, including uroplakin receptors that coat transitional epithelial cells of the bladder [37]. The collective products of the *fimG* and *fimF* genes with the FimH adhesin combine to form the fimbrillar tip found at the end of fimbriae. FimC is a periplasmic chaperone protein which

prevents premature interactions between fimbrial subunits. FimB and FimE are recombinases that control the expression of type 1 fimbriae. A type 1 fimbriated bacterium typically expresses approximately 500 fimbriae per cell [32].

Type 1 fimbriae are proven VFs for *E. coli* in the urinary tract. An *E. coli* strain was selected that was isolated previously from a patient with a UTI and was shown to be capable of causing infections in the murine model of UTI. A mutation was introduced into this strain within the *fimH* adhesion gene [38]. The *fimH* mutant was found to infect the mouse model in significantly lower numbers (1-3 logs less) when compared with mice similarly infected with the wild-type parent strain. Finally, the *fimH* defect was complemented in the mutant strain by placing a functional copy of the *fimH* gene on a plasmid and transforming the mutant with this plasmid construct. The complemented strain was found to infect the mouse urinary tract in numbers determined to be statistically similar to the original wild-type strain, thus demonstrating successful complementation of the former defect in type 1 fimbriae. Muley et al [39] proposed a novel role of type 1 fimbriae in the pathogenesis of UTI that UPEC use type 1 pili to attach to the bladder mucosa, induce apoptosis of host cells, and facilitate invasion and colonization of the damaged tissue. Therefore, *fimH* gene is one of the target genes of VF that was included this study.

1.2) P fimbriae

Bacteria expressing P fimbriae agglutinate human type O erythrocytes, and the hemagglutination was not inhibited by mannose (MRHA); this pattern of agglutination differentiated this new fimbrial type from type 1 fimbriae, which display mannose-sensitive hemagglutination [32].

P fimbriae are encoded by the *pap* (pyelonephritis-associated pili) operon [40]. A number of studies have correlated the presence of P fimbriae and virulent uropathogenic strains [41]. *E. coli* from patients with pyelonephritis are six times as likely to possess P fimbriae as strains from the feces of controls. Approximately 80% of pyelonephritis strains have P fimbriae. The prevalence of P fimbriae expression is strongly associated with the severity of the infection. Studies of humans with UTI demonstrating expression of P fimbriae by *E. coli* in urine [42] and antibodies to P fimbriae in serum [43] indicate that *E. coli* produce and display P fimbriae *in vivo* in infected patients. In addition, a few studies of P fimbriae in canine UTIs have demonstrated a role of pathogenesis. Surveys of canine UTI isolates demonstrated that canine ExPEC strains exhibit a high prevalence of virulence-associated traits typical of human UPEC strains, including digalactoside-binding P fimbriae, hemolysin, and the O4 and O6 somatic antigens [44]. Thus, these investigators proposed that canine UPEC strains might pose an infectious threat to humans.

The expression of P fimbriae by pyelonephrogenic strains was examined to explain the virulence of these strains in the urinary tract. The adherence phenotype conferred by this cell surface structure explained the affinity of these strains for uroepithelium of the ureter. Therefore, intense investigation of the role of P fimbriae in the pathogenesis of acute pyelonephritis was undertaken [32]. Several studies have implicated P fimbriae and hemolysin in the pathogenesis of acute pyelonephritis. Adherence by P fimbriae of UPEC plays at best a role in the development of acute pyelonephritis in the mouse model. P fimbrial gene (*pap*) are preferentially found in urovirulent strains, may serve as a marker for the presence of additional genes that confer virulence, and themselves contribute to virulence [45].

Strains that carry P fimbriae can be categorized by which of the three PapG adhesins are expressed at the tip of the fimbria. PapG I was the first for which the gene was cloned but is not

common in human isolates, PapG II is the most common of the adhesins, and PapG III is found in some cystitis isolates. The three adhesins recognize slightly different portions of the Gal-Gal disaccharide-containing glycosphingolipids [32].

1.3) other fimbriae

Dr adhesin

The Dr adhesin family is composed of fimbrial and afimbrial structures on the surface of *E. coli* that bind to the Dr blood group antigen, a portion of the decay-accelerating factor, which is a membrane protein that prevents the host defense. Within the urinary tract, Dr adhesins bind to the bladder epithelium and type IV collagen on basement membranes. At the present, the role that Dr adhesin plays in the pathogenesis of UTI is unclear. These adhesins are present are minority of UTI strains [32].

F1C fimbriae and S fimbriae family

A family of *E. coli* fimbriae that includes S and F1C fimbriae has been linked to UTI and other extraintestinal infections, particularly neonatal meningitis [32]. However, a few studies reported that F1C fimbriae are more common among pyelonephritis and cystitis strains than among fecal control strains of *E. coli* [7]. F1C fimbriae are expressed *in vivo*, as determined by immunofluorescence in the urine of patients with UTIs. Results of experiments using true isogenic mutants in an attempt to determine the role of these in UTI have not been reported.

2.) Toxins

2.1) α-hemolysin

E. coli hemolysin is the prototypic member of the family of RTX (repeats-in-toxin) toxins. The *hly* operon (*hlyCABD*) is often located adjacent to the P fimbrial genes on the same pathogenicity island of UPEC strains [46]. Therefore, it is not surprising that UPEC are more likely to have the *hly* genes than are fecal strains.

The *hly* genes encode the proteins required for synthesis and secretion of hemolysin. The *hlyA* gene encodes the prohemolysin protein. Generation of the mature and active form of the toxin is followed by fatty acylation of the prohemolysin protein by HlyC and an acyl carrier protein. Hemolysin is secreted to the extracellular milieu by type I secretion system. The export system consists of an ATP-binding cytoplasmic membrane protein, HlyB; a cytoplasmic membrane protein with a large periplasmic domain, HlyD; and an outer membrane protein. HlyB and HlyD form a complex to which hemolysin binds. Hemolysin binding then triggers a comformational change that brings in TolC which forms a channel through the outer membrane [47].

At high doses, hemolysin is cytotoxic not only to erythrocytes but also to a variety of nucleated cell types, including leukocytes, fibroblasts, and more relevant to UTI, uroepithelial cells. Hemolysin also plays a role in invasion of the renal parenchyma by destruction of the epithelial barrier. *In vivo*, hemolysin can stimulate production of cytokines which can lead to inflammatory responses. Anti-hemolysin antibodies are induced during UTI, indicating that hemolysin is expressed *in vivo* [48]. Hemolysin activity against leukocytes may play role in pathogenesis.

2.2) Cytotoxic necrotizing factor 1 (CNF1)

CNF1 is found commonly in strains of *E. coli* isolated from patients with UTI but nearly always in association with hemolysin [49], with which it is genetically linked. CNF1 induces the formation of actin stress fibers and membrane ruffling in HEp-2 cells. The cytoskeleton rearrangement results from constitute activation of members of the Rho family of small GTP-binding proteins due to deamination of glutamine 63 of Rho protein into glutamic acid by CNF1 [50].

CNF1 plays a potential role in the pathogenesis UPEC. CNF1 was shown to increase adherence of PMNs to monolayers and decrease their phagocytic effect. CNF1 also causes apoptosis in the bladder cell line that might explain the exfoliation of bladder epithelial cells after infection with UPEC. Although these effects together suggest that CNF1 may play a role in UTI, an isogenic mutant of *cnf1* was not different from its wild-type parent UPEC strain CFT073 in its ability to colonize or cause inflammation in a murine model of UTI [50], and thus the role of CNF1 in UTI remains unconfirmed.

1.3 The link between virulence and resistance

Subsequent mutability in response to antimicrobial therapy might facilitate emergent virulence along with resistance. Most VF genes in *E. coli* are clustered within chromosomal pathogenicity-associated islands [51] or are carried on plasmids, both of which also carry antimicrobial resistance genes. In general, studies often focus on the loss of resistance as virulence is acquired. For example, extraintestinal strains most able to infect an immunocompetent host tend to be susceptible to fluoroquinolones prior to antimicrobial

exposure [52]. However, high-virulence group B2-derived isolates can acquire resistance to a number of drugs. For *E. coli,* transition from fluoroquinolone susceptible to resistant rarely is accompanied by a loss of virulence factor genes *in vitro* [53]. Among the reasons that stain type (ST) 131 presents a public health risk is that it is not only virulent, but that it is frequently co-resistant to both fluoroquinolones and beta-lactams. Further, resistance is carried on conjugative plasmids and thus is mobile [54] [55]. Fluoroquinolone resistance involves three kinds of plasmid-mediated quinolone resistance (PMQR) genes: *qnr* (directly inhibition [56], *aac(6')-Ib-cr* (encoding enzymatic destruction [57]; and *qepA* (efflux pump overexpression resulting in multidrug resistance; [58]. Accompanying genes for beta-lactam resistance are co carried, reflecting CTX-M enzymes which include extended-spectrum β-lactamases (ESBL) such as CTX-M-15 [59]. This association is particularly worrisome because of the potential for rapid spread of these virulent, multi-drug resistant isolates among community and hospital settings. This clone appears to be responsible for an international epidemic [55] and has been documented in dogs and cats in a household in the United States, indicating sharing among household members, including both pets and people [60].

1.4 Antimicrobial resistance

Emerging antimicrobial resistance and especially multi-drug resistance (MDR) is a major public concern in both human and veterinary medicine since resistance contributes to therapeutic failure, and increased patient morbidity and mortality, and health care costs. Thus, the invention of new drug classes or modification of old classes of antimicrobials have been developed over the past five decades. However, bacteria also can rapidly develop new resistance mechanisms. Currently, antimicrobial resistance mechanisms have been reported for all antimicrobials

currently used in practice of both human and veterinary medicine. Most MDR organisms that are clinically significant include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli , Klebsiella pneumoniae,* vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* [61].

1.4.1 Acquisitions of antimicrobial resistance and multidrug resistance

Acquisition of antimicrobial resistance requires two major components: antimicrobial drugs, which inhibit susceptible microorganisms and select the resistant ones; and the genetic resistance determinant in microbes selected by the antimicrobial drugs [62]. Antimicrobial resistance emerges only when the two components come together in a host, which can lead to a clinical problem. Several classes of antimicrobial drugs are essential for the physiological, structural, or metabolic functions of the bacterial cells. Each class has a specific resistance mechanism [62]. Acquired antimicrobial resistance mechanisms can be classified into four categories: 1) reducing or prevention drug penetration into the bacterial cell; 2) increasing efflux pump activity; 3) modification or degradation of antimicrobials; or 4) modification of antimicrobial targets [63].

1.4.2 Mechanisms of antimicrobial resistance in E. coli

Several mechanisms of antimicrobial resistance have been categorized. Molecular mechanisms of bacterial antimicrobial resistance are classified into intrinsic and acquired mechanisms [64]. An intrinsic mechanism is natural to all the members of a specific bacterial group (genus, species or subspecies), and results from structural or biochemical characteristics inherent to the wild-type microorganism. For example, anaerobic bacteria naturally reduce the activity of aminoglycosides because these agents show poor drug penetration into the cell under

anaerobic conditions. Antimicrobial resistance can also be acquired, often through genetic change in a normally susceptible organism. This type of antimicrobial resistance usually leads to a change in the minimum inhibitory concentration (MIC) of an organism. An example of this type is resistance to fluoroquinolones associated with a mutation in the topoisomerase gene [65]. In veterinary practice, the five most common antimicrobials use for treatment of *E. coli* infection are β-lactams, fluoroquinolones, sulfonamides, tetracyclines, phenicols, and aminoglycosides.

1.) β-lactams: penicillins and cephalosporins

The most commonly detected mechanism of β-lactam resistance in *E. coli* is production of β-lactamase enzymes whose genes are encoded on either chromosomes or plasmids [66]. The mobile elements of many β-lactamase genes have played an important role in their spread within and between species. Mobile DNA elements are normally associated with transposons, insertion sequences, integrons, and plasmids [67]. Resistance to early penicillins emerged after selection of strains with chromosomal β-lactamase genes and subsequent emergence of plasmid mediated β-lactamases, principally the TEM, SHV, and OXA enzymes [68]. Modification of β-lactams to prevent compounds from enzymatic degradation has led to selection pressure of expressing mutant *tem* or *shv* genes which encode the extended spectrum β-lactamases (ESBL). Thus, ESBLs can inhibit antimicrobial activity of new generations of β-lactams, including 3rd generation cephalosporins, carbapenems, or monobactams. The chromosomal *ampC* gene of *E. coli* is normally produced at low levels; however, plasmid encoded AmpC enzyme and overexpression of AmpC have been reported in cephalosporins-resistant isolates of *E. coli* [69] [70].

2.) Fluoroquinolones

Fluoroquinolone (FQ) resistance in *E. coli* is mediated by alteration of the target enzymes DNA gyrase and topoisomerase IV [71] [72] or by reduced antimicrobial accumulation by either porin down-regulation or efflux pump overexpression [73] [74]. Mutations within the genes encoding the target proteins occur within defined quinolone resistance determining regions (QRDRs). DNA gyrase is composed of two GyrA and GyrB subunits [75]; topoisomerase IV is composed of two ParC and two ParE subunits [76]. GyrA is the primary target for FQ in *E. coli*, and a number of mutations within QRDR of *gyrA* confer resistance; the most common mutations are substitution of serine 83 of GyrA [77] [72]. Accumulation of mutations with *gyrA* may lead to further mutations within other target protein especially ParC; mutations within GyrB and ParE are less common. Moreover, FQ-resistant clinical isolates of *E. coli* might show reduced accumulation which is associated with overexpression of the *marA* or *soxS* regulons or the AcrAB-TolC efflux system causing multidrug resistance [73] [74] [78].

3.) Sulfonamides/Trimethoprim

The targets of sulfonamides and trimethoprim are dihydropteroate synthase and dihydrofolate reductase, respectively [79] [80]. Resistance is acquired by novel variants of the target enzymes two sulfonamide resistance genes (*sulI* and *sulII*) and over 15 trimethoprim resistance genes (dihydrofolate reductase-*dhfr*); the most common genes in *E. coli* are *dhfrI* and *dhfrII* [79]. Overexpression of chromosomal enzymes can lead to resistance of trimethoprim. The *dhfr* genes can also be transferred by plasmids, transposons, and integrons [79] that might lead to increase prevalence of *E. coli* trimethoprim-resistance.

4.) Tetracyclines

Tetracycline acts as a protein synthesis inhibitor by binding ribosomes. Tetracycline resistance can be mediated by active efflux pumps (the major mechanism), inactivation of the tetracycline molecule activity, and mutation of rRNA (preventing tetracycline from binding to the ribosome). The Tet family of efflux pumps is widely found in gram-negative bacteria, including *E. coli*. The Tet pumps act as antiporters for which a proton is exchanged for a drug compound. The most common resistant genes in *E. coli* are *tetB* and *tetA* genes, although many *tet* genes have also been reported in *E. coli.* The Tet pump genes are readily mobilized and often associated with Tn*10* or a plasmid; thus, tetracycline-resistant *E. coli* is widely and rapidly spread [81].

5.) Aminoglycosides

Most clinically important resistance to aminoglycosides is caused by R-plasmid-specified inactivation enzymes. They are classified as phosphotransferases, acetyltransferases, or adenyltransferases [82]. At least 11 enzymes had been identified, each of which attack hydroxyl or amino groups, thus preventing ribosomal binding. The acetyltransferase enzyme genes often associated with plasmids or transposons [83].

1.4.3 Definitions of MDR, XDR and PDR

Definitions of single drug (SDR), multi drug (MDR), (extreme-multi drug) XDR and (pan drug resistance) PDR strains have been proposed for pathogenic bacteria that are frequently found in healthcare settings (e.g. *E. coli, S. aureus, Enterococcus* spp*., Enterobacteriaceae, Pseudomonas aeruginosa,* and *Acinetobacter* spp.). The use of standard terminology will optimize epidemiological surveillance systems, and facilitate the exchange of information

between the medical community, public health authorities and policy makers in order to promote the prudent use of antimicrobials and other public health measures.

Several definitions for MDR, XDR, and PDR depend on mechanisms of antimicrobial resistance. Classification by chemical structure is useful for establishing chemical structure-antibiotic activity relationships. In general, the molecular core structure of antimicrobials is responsible for drug activity. Addition or deletion of chemical groups leads to various members of the class and influences their spectrum of activity, pharmacodynamics, pharmacokinetics and toxicology. No standard definition have been used to determine SDR, MDR, XDR, and PDR. Mostly, chemical structures for antimicrobial classes (e.g. β-lactams), antimicrobial subclasses (e.g. 3rd generation cephalosporins) or specific antimicrobial agents (e.g. ceftazidime) have been used to define the phenotype of resistance. However, this approach is not always conclusive and makes it difficult to compare results between studies. Therefore, the antimicrobial categories (Table 1.1) which were classified by mechanism of resistance for each organism group has been proposed for the intent of placing antimicrobials phenotype [84]. This study involved antimicrobial agents which are relevant to veterinary use in small animals.

For definition of SDR, MDR, XDR, or PDR, non-susceptibility refers to either a resistant, or intermediate result obtained from *in vitro* antimicrobial susceptibility testing. MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (or bacterial isolates remain susceptible to only one or two categories). PDR is defined as non-susceptibility to all agents in all antimicrobial categories (or no agents tested as susceptible for that organism) [84].

Antimicrobial category	Antimicrobial agent
Aminoglycosides	Gentamicin
	Amikacin
Antipseudomonal penicillins + β-lactamase inhibitors	Ticarcillin-clavulanic acid
Carbapenems	Meropenem
Non-extended spectrum cephalosporins	Cefazolin
	Cephalothin
Extended spectrum cephalosporins (3rd and 4th	Ceftazidime
generation cephalosporins)	Cefotaxime
Cephamycins	Cefoxitin
Fluoroquinolones	Enrofloxacin
	Ciprofloxacin
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole
Penicillins	Ampicillin
Penicillins+ β-lactamase inhibitors	Amoxicillin + clavulanic acid
Tetracyclines	Doxycyline

Table 1.1 *Enterobacteriaceae*; antimicrobial categories and agents used to define MDR, XDR, and PDR [84].

1.5 Antimicrobial resistance risk assessment

The question of the impact of veterinary antimicrobial use on human health has been controversial. Because of this, antimicrobial resistance risk assessment (ARRA) is being used to

estimate the magnitude of the link between the veterinary use of antimicrobials and the emergence of resistant organisms in human [85].

1.5.1 Methodological considerations of ARRA

There are three main approaches for the ARRA: qualitative, semi-quantitative and quantitative, and no golden rule has been developed. When choosing which method to adopt, the most important criterion is whether the assessment is fit for the purpose.

1.) Qualitative risk assessment

In qualitative risk assessment, the risks are predicted using descriptive terms. The data requirement for a qualitative risk assessment are identical to those needed for a quantitative model. A qualitative risk assessment requires fewer mathematical resources and therefore provides a faster assessment of the risk in comparison to their quantitative counterparts. Indeed, qualitative assessment are often carried out as a pre-cursor to a quantitative assessment as the process can indicate whether or not a further quantitative assessment is required.

Two methods are used to combine qualitative probabilities or parameter. The first method, non-matrix method, involves the risk assessor considering the probabilities/parameters to be combined, while taking into account the variability associated with the data, including data quality, and providing assessment. The non-matrix method allows the risk assessor to describe qualitatively the uncertainty and variability throughout the assessment.

The second method used to combine risks within a qualitative assessment is commonly referred to as the ‘matrix method’. Many risk assessors and managers prefer this method because it provides a structured approach for the combining of qualitative risks. This method reduces the level of subjectivity associated with the assessment. However, this approach

based upon the risks (probabilities). In addition, within a risk assessment the probabilities/parameters can be additive or multiplicative and could involve combining multiple probabilities and integers. In practices, it is important when using a qualitative approach that there is clear definition of the terms used, and the matrix is provided, making sure that it respects rules of probabilities calculations.

2.) Semi-quantitative risk assessment

Semi-quantitative analysis (also known as risk ranking), a score is assigned to each of the steps in the risk pathway, for example 1-10. The semi-quantitative method offers a formalized approach. Compared to qualitative risk assessment, it has a higher degree of resolution due to a larger number of possible outcomes. An advantage of this approach is that semi-quantitative risk assessments require less time and resources compared to quantitative models since risks are being ranked so multiple models would have to be developed. However, the interpretation of the allocated scores and/or combined scores is often the subject of great debate.

3.) Quantitative risk assessment

Using mathematical modelling techniques, quantitative risk assessments can be developed which provide a numerical estimate of risk. Two methods can be used: deterministic and stochastic. In a deterministic model, point values are used to parameterize the risk pathway. Combining these parameters provides a point estimate, but the uncertainty or variability associated with the estimate is not implicitly characterized within the assessment. Changes in parameter values allow scenarios to be investigated, and this information is very useful for risk managers.

1.5.2 Data for ARRA: requirements and sources

The availability and quality of data used in an ARRA is absolutely critical. However, there is no standard list of data requirement for ARRA. Such data requirements will differ between risk assessments, due to the risk question posed, scope, and required resolution. The task of developing an ARRA is complicated since the model needs to take into account the presence of the organism, and the antimicrobial of interest. The resistance is attributed to the use of the antimicrobial in the animal species of interest. This makes the data requirements large because sampling schemes for antimicrobial resistance are focused on assessing presence/absence of the resistant organism. In addition, common data deficiencies encountered in ARRA relate to temporal and regional differences that exist in the microbiological methods used to identify bacteria and for susceptibility testing and interpretation, because such methodological differences make it difficult to combine data from different studies. Therefore, harmonization of microbiological and sampling methods, both within and between countries and between veterinary and human medicine are required to ARRA in epidemiological studies.

1.5.3 Epidemiology: a tool for the assessment of risk

Epidemiological investigations can provide strong evidence linking exposure to the incidence of infection or disease in a population. They can provide estimates of the magnitude of risk related to a particular level of exposure or dose and so can be used in the evaluation of appropriate microbiological quality guideline levels or standards. Epidemiological methods can quantify the probability that observed relationships occurred by chance factors and they also have the potential to control for other risk factors and/or confounders of the outcome illness

being studied. Epidemiological studies used for the evaluation or setting of guidelines must be of high quality, so that there is confidence in the validity of the results.

The basic measures of disease frequency in each population are described by using the prevalence rate (which is the proportion of the population that has the disease at a specific point in time) or the incidence rate (the number of new cases of disease per unit of person-time). Measuring the difference between disease frequencies in the exposed and control populations is usually done using a relative measure. The relative risk (RR) estimates the magnitude of an association between exposure and disease. It indicates the likelihood of developing the disease in the exposed group relative to those who are not exposed. The odds ratio (OR) is the ratio of the odds of exposure among the cases to the odds in favor of exposure among the controls. If the disease is rare, the odds ratio will approximate the relative risk. Where multivariate analysis is carried out (a technique that allows an assessment of the association between exposure and disease, while taking into account other risk factors that may be confounding factors) the odds ratios is the relative measure normally calculated.

1.5.4 Surveillance programs and the role of diagnostic laboratories

The seriousness of the antimicrobial resistance threat has prompted many organizations to initiate surveillance programs, which include bacteria of animal origin. These programs provide a tool to globally assess the extent of the problem, to follow its evolution over time, and to evaluate the effectiveness of control measures. Such systems include the National Antimicrobial Resistance Monitoring System (NARMS) in the United States, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMRP). These

programs are the national surveillance programs of foodborne bacteria from humans, retail meats, and animals. In the pet animals, very few surveillance programs obtain antimicrobial susceptibility data from bacterial pathogens of animals. Surveillance programs include collection of data on antimicrobial use and linkage of the latter with the evolution of resistance. A few studies reported the prevalence of antimicrobial resistance in animal origin [86]. However, because of geographically local and temporarily limited nature of these studies and their different sampling and susceptibility testing methodologies, it is difficult to delineate reliable conclusion on the global antimicrobial resistance situation in veterinary medicine. Although the Clinical and Laboratory Standard Institute (CLSI) has been developing veterinary standard for susceptibility testing methodologies, many veterinary laboratories do not strictly follow these standards. To date, veterinary diagnostic laboratories have used human CLSI interpretive standards as the breakpoints of MIC; however, the newer standards included criteria that are veterinary-specific. Because veterinary-specific criteria is not available for some antimicrobials including amikacin, chloramphenicol, etc.; these drugs should be interpreted cautiously, and ideally in the context of pharmacokinetics for each of these drugs in dogs and cats.

Susceptibility testing of clinical isolates is a cornerstone for prudent use of antimicrobials and for an adequate management of single clinical cases. Unfortunately, microbiological analysis and susceptibility testing are still frequently performed only when a problem has not been resolved by empirical antimicrobial therapy.

1.5.5 Antimicrobial susceptibility testing methods and interpretation of results

Antimicrobial susceptibility testing (AST) is evaluation of the ability of a specific organism to grow in the presence of a particular drug in vitro and is performed using guidelines

established by the Clinical and Laboratory Standards Institute [87]. The goal of AST is to predict the clinical success or failure of the antibiotic being tested against a particular organism.

1) Disk diffusion versus dilution technique

The disk diffusion method involves disks that contains a known amount of drug. The drug diffuses from the disk into the agar, the concentration in the agar correlates with the MIC of the drug. A zone of no microbial growth is measured around the disk. Because the concentration of drug decreases with the zone diameter from the disk, the larger the zone, the lower the concentration of drug needed to inhibit the microbial growth and the more likely effective drug concentrations will be achieved at the site of the infection. Zone size is essential for an organism to be considered susceptible as opposed to resistant. Disk diffusion results are reported as S, I, R; therefore, it is considered as qualitative (Figure 1.4) [87].

The broth dilution method provides quantitative and serves as a gold-standard to other methods. For each tested drug, tubes or wells (for broth microdilution) are spiked with concentrations of the drug, with the highest concentration generally below or above the CLSI threshold of susceptibility (susceptible/ resistant MIC breakpoint). Test tubes contain serially 2-fold diluted concentrations of the drug. As such, MICs are reported as logarithmic numbers (i.e. 0.25, 0.5, 1, 2, 4, 8, 16… Figure 1.5).

A) **B)**

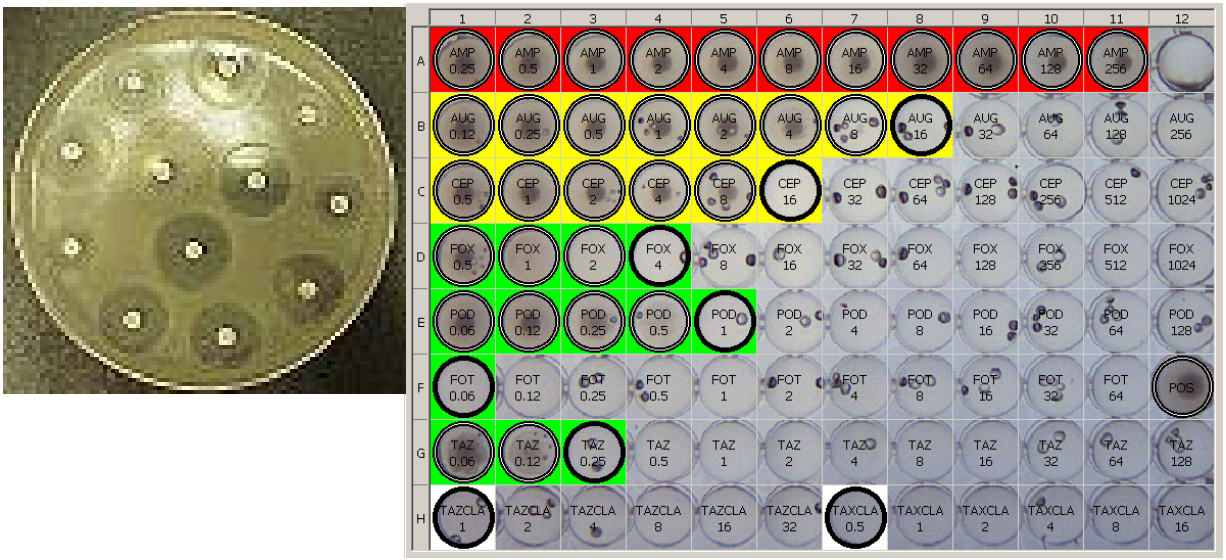

Figure 1.4 Antimicrobial susceptibility method A) Disk diffusion. The diffusion of antimicrobial agent from a disk through a solid medium with a defined concentration of a bacterium. A zone of inhibition forms when the concentration of the drug becomes too dilute to inhibit the growth of the bacterium. B) Broth microdilution is using antimicrobial agents of known potency in progressive two-fold dilutions. Results are determined by the growth of the bacterium in the presence of varying concentration of the tested drug. The MIC is usually read as the first well with no obvious growth.

2) Antimicrobial susceptibility testing interpretation

Data are reported in the term of MIC, which is the lowest concentration of an antibiotic that inhibits visible growth of a microorganism [87]. Although AST results are generally quite useful in narrowing the antibiotic regimen, AST has some limitations that should be considered. 1) The site of infection: Clinical laboratories may provide different AST interpretations for different sites of infection (eg, urine VS non-urine). 2) Bacterial enzyme expression: Enzyme

expressed in vivo can inactivate antimicrobial agents to which the organism shows in vitro susceptibility. Extended-spectrum β-lactamases (ESBLs) in *Enterobacteriaceae* are enzymes that mediate resistance to almost all β-lactam agents except carbapenems (eg, meropenem or imipenem). ESBLs can be hard to detect because they have different levels of in vitro activity against various cephalosporins. The production of ESBL should be suspected when treatment with β-lactams fails despite apparent in vitro susceptibility. This should lead to additional testing, which involves growing bacteria with a 3rd generation cephalosporin and in combination with clavulanic acid (a β-lactamase inhibitor). If bacterial inhibition is enhanced with the addition of clavulanic acid, these bacteria indicates the productions of ESBL [88].

3) Population pharmacodynamics statistics

Population statistics generated from MICs provide useful information that describe the population distribution include the range of the lowest to the highest concentration of tested drug, mode, median (the 50th percentile or MIC_{50}), and MIC_{90} (the 90th percentile) (Figure 1.5). The distribution of the MICs of bacteria for antimicrobial is helpful to identify emergence resistance. For example, the distribution of *E. coli* for enrofloxacin (Figure 1.5) is bimodal, representing two different populations. The most isolates in the first population are characterized by an MIC below the susceptible threshold of susceptibility (S-MIC_{BP}). Moreover, the distribution demonstrates that susceptible isolates are close to S-$MIC_{BP.}$ The population of the second mode is higher than the upper S-MIC_{BP}, probably represents isolates previously exposed to antimicrobials. Finally, increasing MICs determined from sequential cultures of the same organisms with recurrent infection might indicate emerging resistance. Also, comparison of MIC_{90} of a sample population across time can reveal emerging resistance.

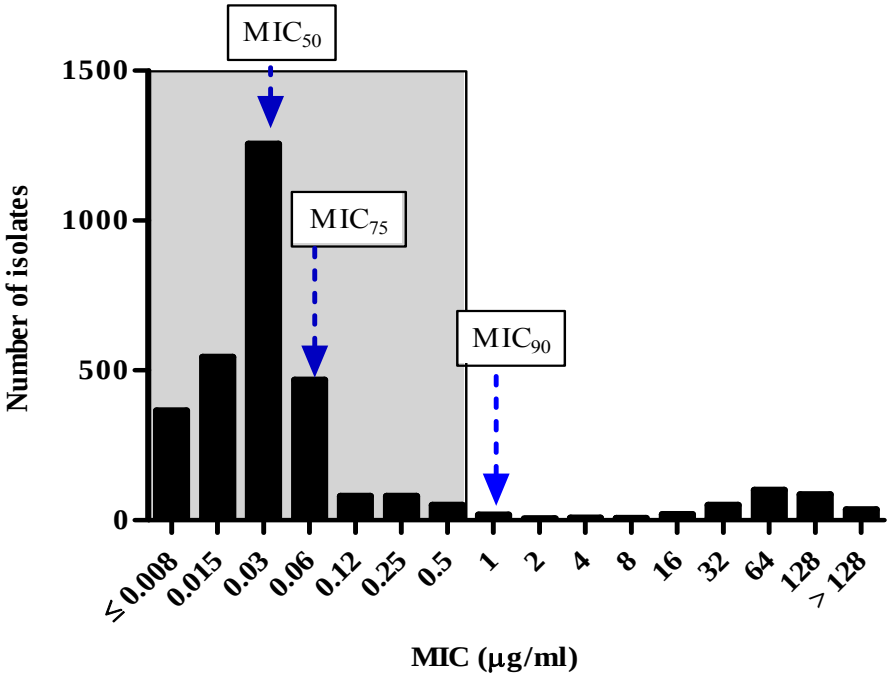

Figure 1.5 An example of a population MIC distribution plot based on *E. coli* cultured from dogs and cats (*n*= 3013 isolates). The shaded area represent the susceptible MICs, based on CLSI interpretive standards [87]. The distribution of all the isolates data are bimodal, characterized by a second population of resistant isolates.

1.6 Research objectives

This dissertation is composed of four studies conducted in clinical *E. coli* isolates from dogs and cats. The main scope was focused on epidemiology of antimicrobial resistance and virulence of *E. coli* had also been a key component of the studies.

Chapter II is an epidemiological study to investigate the current pattern of antimicrobial susceptibility for presumed clinical *E. coli* isolates in dogs or cats. Isolates were collected from six geographical regions in the US between May 2008 and January 2013. *E. coli* Isolates were phenotypically characterized based on the susceptibility to 16 antimicrobial agents by broth microdilution technique according to CLSI guidelines and interpretive standards.

Chapter III is to investigate the pattern of veterinary antimicrobial prescribing behaviors and to evaluate the appropriateness of use compared with prudent use guidelines. A questionnaire was sent to veterinarians throughout the US submitting dog or cat *E. coli* culture samples. Information was collected regarding clinic demographics, patient signalment, the infection itself, and the antimicrobial intervention. Descriptive statistical analyses were performed to assess the pattern of antimicrobial use. Multinomial logistic regression identified factors associated with antimicrobial prescriptions.

To reduce the unnecessary use of antimicrobials, the virulence is a key component to characterize pathogenic *E. coli* in term of severity of diseases. Thus, chapter IV described the association among the severity of *E. coli* infection in the urinary tract, virulence profiles, and patterns of antimicrobial resistance. Virulence profiles of uropathogenic *E. coli* were characterized by phylogenetic group and the presence of virulence genes. Since phylotype B2, D isolates (more virulence) were more susceptible to antimicrobials that in phylogroup A, B1 (less virulent isolate), the clinical decision of antimicrobial therapy should be based on virulence profiles.

Chapter V is to characterize canine UPEC in order to identify the incidence of asymptomatic bacteriuria (ABU) and unique genes that might discriminate (diagnose) ABU from non-ABU by using principal component analysis and linear discriminate analysis. This study was designed to help us establish the diagnostic model that convincing veterinary practitioners to not treat ABU with antimicrobials requires virulence profiles evidence.

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CHAPTER 2

ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF CLINICAL *ESCHERICHIA COLI* ISOLATES FROM DOGS AND CATS IN THE UNITED STATES

2.1 Introduction

Emerging antimicrobial resistance is a major public concern in both human and veterinary medicine. Resistance contributes to therapeutic failure, increased patient morbidity and mortality, and health care costs [1]. *E. coli* also is a reasonable sentinel microbe for investigation of current trends in antimicrobial resistance in pets because of its ubiquitous environmental presence, its importance in disease and the ease by which it develops antimicrobial resistance [2].

Antimicrobial resistance genes can be horizontally transferred via mobile gene elements [3]. *E. coli* in particular is able to transfer antimicrobial resistance gene between microorganisms [4] and selected multidrug-resistant (MDR) *E. coli* strains become epidemic [5] [6]. Furthermore, outbreaks of virulent and resistant isolates such as strain O15:K52:H1 in humans have been associated with community-acquired cystitis, pyelonephritis, and septicemia in South London in 1987 and 1988 [5] and urinary tract infection (UTIs) in Europe [7]. Importantly, ExPEC isolates from dogs and humans within households are phylogenetically related and share common virulence genes, suggesting a potential for cross-species transmission or co-infection [8]. Because the number of household dogs and cats in the US has dramatically increased [9], the risk of a pet and owner sharing the same strain of a pathogen is a compelling reason for the monitoring of *E. coli* virulence or antimicrobial resistance in pets.

Programs intended to monitor emerging antimicrobial resistance in companion animals have not been implemented. Although limited data has been collected in Veterinary Teaching Hospitals, the applicability of such data may not be relevant to the general veterinary population [10] [11]. Preemptive surveillance of canine and feline pathogenic *E. coli* is indicated to establish current resistance or susceptibility patterns to facilitate empirical antimicrobial treatment as well as lay a foundation for the study of potential risk factors associated with the emergence of MDR *E. coli* across the country.

The purposes of this chapter were to describe the current patterns of antimicrobial susceptibility and resistance for feline and canine *E. coli* pathogens in the United States, and to characterize demographic and clinical features with associated *E. coli* antimicrobial resistance. Further, this study sought to identify factors that could predict the risk of resistance in such pathogens. We hypothesized that regarding small animal *E. coli* pathogens exhibiting multidrug resistance: the overall proportion of resistance in the United States approximated 40%; the proportion of resistant isolates was greatest in the south compared to other geographic regions.

2.2 Materials and methods

2.2.1 Source of isolates

Isolates (n=3172) of *E. coli* were provided by a commercial diagnostic laboratory (CDL) to the clinical pharmacology laboratory (CPL) at Auburn University. Samples had been submitted to the CDL from January 2008 through January 2013 by veterinary practitioners after collection from dogs or cats with presumed naturally-occurring infection. Isolates were sub-cultured by the CDL on BBL™ Trypticase™ soy agar slants (BD Diagnostic Systems, Sparks, MD) and shipped to the CPL overnight at room temperature. Although susceptibility testing had

been performed by the CDL prior to submission to the CPL, this data was not available to the study investigators. Select patient demographical data was available for each isolate.

2.2.2 Sample collection and susceptibility testing

On receipt, each isolate was prepared for susceptibility testing using procedures and guidelines promulgated by the CLSI [12]. Samples were confirmed to be *E. coli* and tested for contamination with other bacteria by culture on CHROMagar™ Orientation (BD Diagnostic Systems, Sparks, MD). Contaminated samples were excluded from this study. Colonies subjected to susceptibility testing were subcultured directly from CHROMagar™ Orientation by streaking on to a Trypticase™ soy agar (BD Diagnostic Systems, Sparks, MD) plate. Inocula were prepared by suspending growth from overnight cultures in sterile saline to turbidity of approximately 0.5 McFarland turbidity standard (BD Diagnostic Systems, Sparks, MD). Final inocula contained 2 to 7 x 10^5 CFU/ml. The suspension was used to inoculate 96-well custom-made plates (TREK Diagnostic Systems, Cleveland, OH). The plates were incubated at 37°C in cation-adjusted Mueller Hinton II Broth (BD Diagnostic Systems, Sparks, MD) and read at 18 hours. Susceptibility testing was performed using 17 antimicrobials, representing 6 drug classes (Table 2.1). These 17 drugs also were classified into 12 antimicrobial categories based on the type of resistance, including penicillins: ampicillin (AMP, also serving as a model for amoxicillin); penicillins+β-lactam inhibitors: amoxicillin-clavulanic acid (AMC, also serving as the model for ampicillin-sulbactam); anti-pseudomonal+β-lactam inhibitors: ticarcillin-clavulanic acid (TIM); non-extended spectrum cephalosporins (1st generation cephalosporins): cephalothin (CEP) (serving as a model for cephalexin), cefazolin (CEF); extended-spectrum cephalosporins (3rd and 4th generation cephalosporins): cefotaxime (CTX), cefpodoxime (CPD) and ceftazidime (CAZ); cephamycins: cefoxitin (FOX); carbapenems: meropenem (MEM);

tetracyclines: doxycycline (DOX); phenicols: chloramphenicol (CHL); fluoroquinolones: enrofloxacin (ENR) and ciprofloxacin (CIP); aminoglycosides: gentamicin (GEN), amikacin (AMK); and folate pathway inhibitors: trimethoprim-sulfamethoxazole (SXT) (Table 1; Magiorakos et al., 2012). Concentrations tested were at least 2-fold dilutions above and below the susceptible and resistant MIC breakpoints, respectively, and ranged from 0.008 to 0.5 µg/ ml below and 128 to 1024 µg/ ml (Table 2.1), depending on the drug (CLSI, 2013). For quality control purposes *E. coli* ATCC® 25922 (American Tissue Cell Culture, Manassas, VA) were tested with each run.

2.2.3 Interpretation of susceptibility testing results

a.) Pharmacodynamic data or level of resistance (MIC)

The lowest concentration of antimicrobial tested that yielded no growth was recorded as the MIC using the SENSITITER1 VIZION system (TREK Sensititre, Cleveland, OH). Each isolate was designated as resistant "R" (MIC ≥ the resistant breakpoint), susceptible "S" (MIC ≤ the susceptible breakpoint) or intermediate "I" (MIC between susceptible and resistant breakpoint) to each drug using CLSI guidelines (Table 2.1) [12]. Although the proportions of "intermediate" isolated were recorded, for analyses, isolates designated as "intermediate" were considered "resistant". Isolates for which the MIC was limited by the tested concentrations were designated as ≤ or > the concentration and were interpreted by that MIC for calculation of the geometric (GE) mean. The level of resistance was described for each drug based on the ratio of MIC to the resistant breakpoint MIC (R-MIC_{BP}) for that drug as determined by CLSI [12]. A ratio of R-MIC_{BP} to MIC_{90} of 8 or more was defined as high-level resistance of the isolate for that drug based on criteria suggested by Baquero [13]. Since AMP, AMC, and CEP do not have R-MIC_{BP} for urine samples, the ratio to indicate the high-level resistance was computed by the

ratio of S-MIC_{BP} to MIC_{90}. Low-level resistance was considered to start at the upper limit of the S-MIC_{BP} and to end the point that high-level resistance started [14].

b.) Phenotypic susceptibility testing or type of resistance

Each isolate was categorized in terms of its resistance phenotype as to: no drug (NDR), non-multidrug (SDR), multi-drug (MDR), or extreme drug (XDR). SDR was defined as resistance to 1 or 2 of the previously described 12 antimicrobial categories (e.g., an isolate expressing resistance to both AMC and CEP was considered to be an SDR isolate). Multi-drug resistance was defined as resistance to three categories [15], whereas XDR was defined as resistance to all except 2 or fewer antimicrobial categories [15].

2.2.4 Data collection

The information accompanying each isolate provided by the CDL was recorded, including: signalment (species, gender, and age), isolate tissue source, and zip code of sample origin. Six geographical locations of isolate origin were identified based on the zip code of the sample origin [16]: northwest (NW), southwest (SW), north central (NC), south central (SC), northeast (NE), and southeast (SE) (Fig. 2.1).

2.2.5 Statistical analyses

All data were manipulated and analyzed by using SAS software 9.3 (SAS Institute Inc., Cary, NC). All tests of significance were two-tailed ($P<0.05$). Population distribution histograms were performed using GraphPad Prism version 5.04 for Windows, GraphPad Software (La Jolla, CA).

a.) Descriptive Pharmacodynamic data

MIC statistics calculated for each drug included the mode, mean (calculated as geometric mean to allow for non-normality of data), range, mode, median (MIC_{50}), 75th (MIC_{75}) and 90th

percentiles (MIC_{90}). Descriptive MIC statistics were calculated for all isolates, for each species, and for each of the 6 geographical regions.

b.) Percent of Resistant Isolates

For each drug, isolates were designated as either susceptible or resistant (binary response). Resistance percentages (R%) and 95% confidence intervals (CI) were calculated to examine the bivariate association. The Mantel-Haenszel chi-square test [17] within PROC FREQ was used to assess trends in the relationship between R% and geographical regions, species, gender, and age. All tests of significance were two-tailed ($P<0.05$). To compare resistance rates among antimicrobials, a multiple comparisons test for proportions was implemented using the COMPPROP macro in SAS which is analogous to a Tukey-type multiple comparison test as used with one-way analysis of variance and considered significance at $P<0.05$ [18].

c.) The proportion of phenotypic resistance

The proportion of each isolate expressing phenotypic antimicrobial resistance to each drug was described by geographical regions, and signalment including species, gender and infection location. The chi-square test [17] within PROC FREQ was used for proportion comparison among types of phenotypic resistance.

d.) Identifying risk factors

A multinomial logistic regression was implemented to allow analysis of correlated factors for R% and type of resistance, using the Logistic procedure in SAS® [19]. The type of resistance; including SDR, MDR, and XDR were nominal response variables and then the multinomial logistic regression was implemented in PROC LOGISTIC to identify risk factors with odd ratio [20]. We selected a list of candidate variables that would be examined for their association with R% and types of resistance (SDR, MDR, and XDR). These variables included

geographical regions, demographic characteristics (species, gender, and age), and infection location (urine and non-urine). Each of the variables was examined for its association with R% and prevalence of NDR, SDR, MDR, and XDR. All variables are categorical; therefore, a Chi-squared test was used to determine the statistical significance of the association between the variables and type of resistance. Variables that were significantly associated with types of resistance with a significance level of $P < 0.25$ were selected by forward selection for possible inclusion in multivariate logistic regression models. We chose $P=0.25$ as the threshold for including variables in the multivariable model because this has been suggested as an appropriate threshold. Second, the variables of final models were selected with significance $P<0.05$. Model parameters were estimated by the maximum-likelihood method by using Schwarz Criterion for selection. From these estimates, odds ratios (OR) with 95% CI were computed.

2.3 Results

2.3.1 Isolate Description

Of the 3172 isolates received, 2681 indicated the geographical region of origin (Figure 2.1); the regions that were the most and least represented were SW (n=774; 28.9%) and NW (n=107; 4.0%) (*P<0.001*), respectively. Regarding species, 2392 (75.4% CI 73.9-76.9%) and 780 (24.6% CI 23.1-26.1%) were collected from dogs and cats, respectively. For dogs, 69.1% (CI 67.1-71.1) were female and 31.0% (CI 29.0-33.1) male. For cats, 74.9% were females and 26.5% were males (1.1% was listed as unknown). Age distributions for both dogs and cats are illustrated in Figure 2.2. The median ages of dogs and cats were 7.6-10 and 12.6-15 years, respectively. In dogs, the 2 most common age groups were 7.6-10 years (n=446; 22.4%) and 10.1-12.5 years (n=452; 22.7%) and the least common was over 15 years (n=56; 2.8%;

P<0.0001). The age groups of dogs and cats' population were significantly different. For cats, the age groups over 15 years (n=186; 28.6%) and up to 1 year old (n=23; 3.5%) were the most and least common (*P<0.001*), respectively. The proportion of geriatric cats or age group of 12.6-15 (24.6%) and > 15 years (28.6%) were significantly higher than those in dogs (10.9%, 2.8%, respectively; *P<0.05)*.

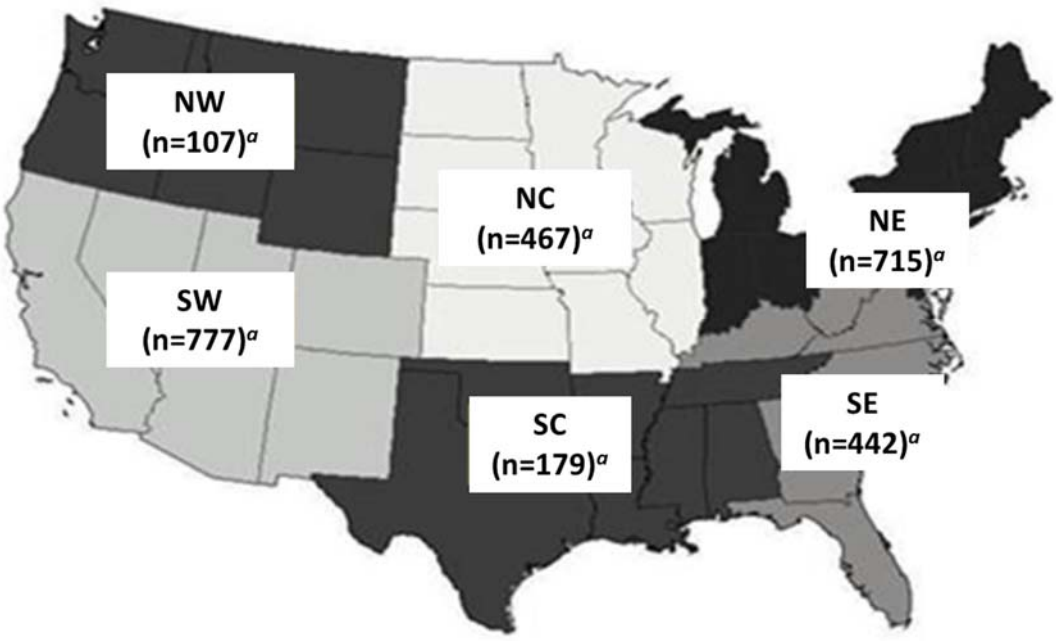

Figure 2.1 The six geographical regions from which isolates were collected based on the zip code of isolate origin. [a]Number in parentheses is the total number of *E. coli* isolates collected from that region.

NC, North central; NE, Northeast, NW, Northwest; SE, Southeast; SC, South central; SW, Southwest.

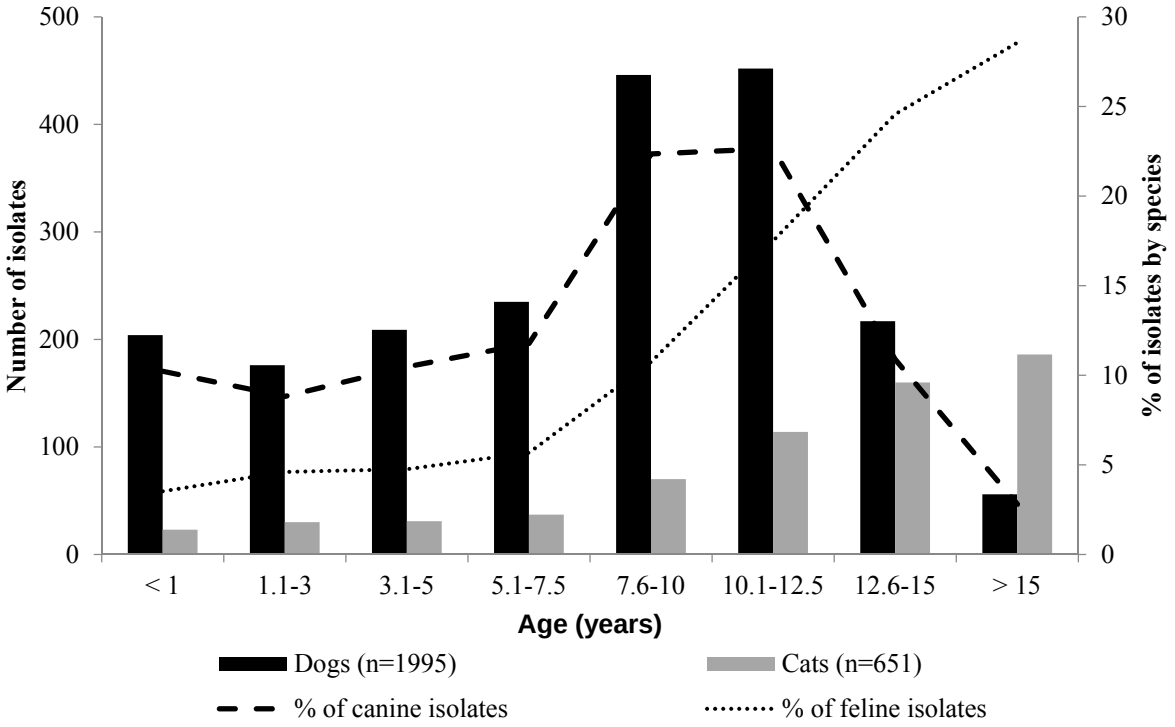

Figure 2.2 Age population distribution for dogs and cats from which *E. coli* isolates were collected. The left Y axis represents the numbers of isolates (frequency) for each age group (X axis). The right Y axis represents the percentages of isolates by each specie. Comparison proportions between canine and feline isolates based on the age population distribution which *E. coli* isolates were collected. The proportions of feline compared with canine isolates with geriatric age (12.6-15, > 15 years) is greater ($P < 0.05$) than those in younger age groups (<10 years).

Isolates were collected from 33 different infection sites (Figure 2.3). The far majority of isolates (N=2138; 70.6%) were collected from the urinary tract, with the ear representing a distant second (7.2%). No other body system represented more than 4% of isolates. For non-

urinary isolates, those representing the 10 most common sites included ears, genital areas, skin swab, abscess, upper respiratory tract, wound, lung, body fluid, uterus and oral cavity (Figure 2.3; Table 2.3).

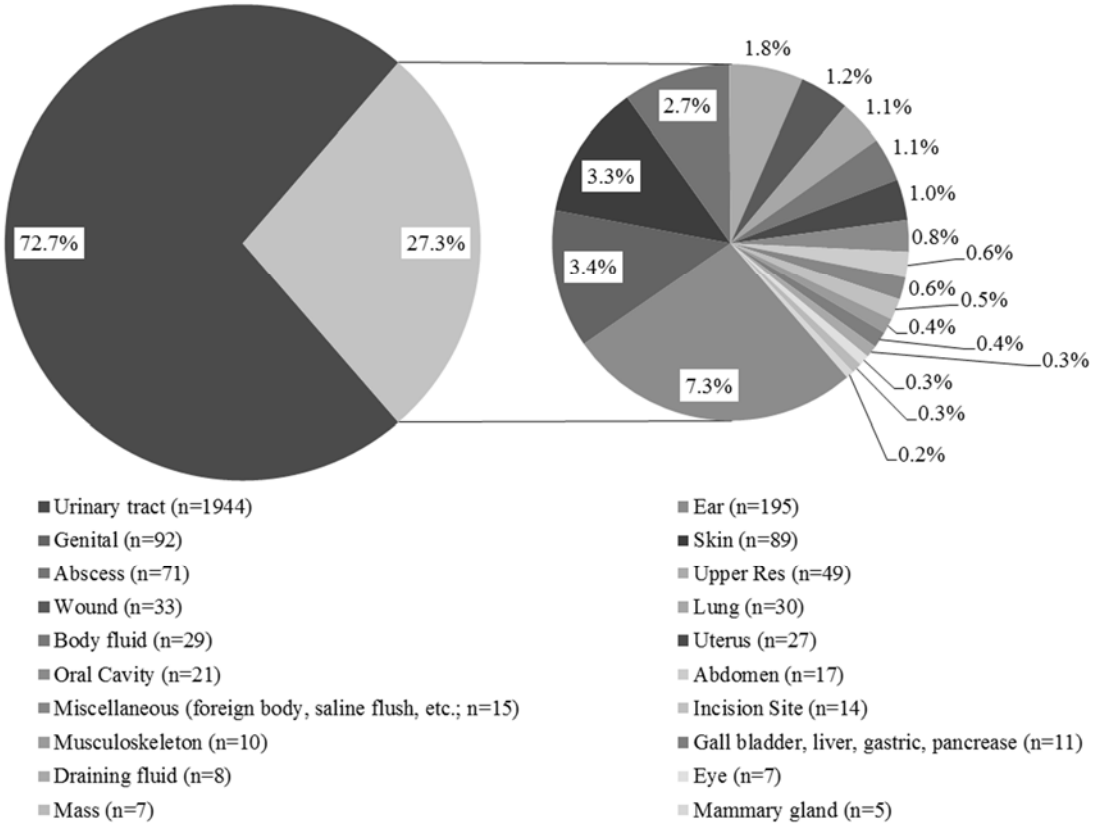

Figure 2.3 The proportion of isolates collected from each body location. The urinary tract was the most common location of infection.

2.3.2 Pharmacodynamic data

MIC statistics for each drug are delineated in Table 2.1 MIC distributions (Figure 2.4) were generally bimodal except for GEN, AMK, and MEM; the second modes of AMP, AMC,

CEF, CEP, CPD, CTX, DOX, FOX, ENR, CIP, and TIM were above the R-MIC_{BP}. The second modes for AMP and CEP were the highest tested concentration; for both of these drugs, the MIC_{75} of AMP, CEF, and CEP was also above the R-MIC_{BP} (Fig 2.4). CEP and DOX was the only antimicrobial for which both first and second modes exceeded the R-MIC_{BP}. The MIC_{90} exceeded the R-MIC_{BP} for AMP, AMC, CEF, CEP, CPD, DOX, ENR, FOX, and TIM whereas MIC_{90} of the remaining drugs were lower than the R-MIC_{BP} (Figure 4). Percentages of high-level resistance of DOX (14%; $P<0.01$), CEP (51%; $P < 0.0001$), AMP (47%; $P < 0.0001$), AMC (34%; $P < 0.0001$), CPD (12%; $P = 0.03$) and ENR (11 %; $P=0.04$) were significantly higher than other antimicrobials (Table 1). For the low-level of resistance, the proportions of total isolates DOX (81%; $P<0.0001$), CHL (57%; $P < 0.0001$), CEP (49%; $P < 0.0001$), AMC (19%; $P = 0.03$), and TIM (18%; $P = 0.03$) were higher than those of other drugs.

Drug	Conc. range tested (µg/mL)	Breakpoint MIC (µg/mL)		Mode	MIC_{50}	MIC_{75}	MIC_{90}	GE mean	Percentages of MIC ratio (MIC:R-MIC_{BP})	
		Susceptible	Resistant						1 - 8	≥ 8
Penicillins										
AMP	0.25-256	≤ 8[a]	n/a	2	4	128	> 256	10.1	5	47[b]
Penicillins+β-lacatamase inhibitors										
AMC	0.12-1024	≤ 8/4[a]	n/a	4	4	8	32	5.2	19	34[b]
Antipseudomonal+β-lacatamase inhibitors										
TIM	0.25-512	≤ 16/2	≥ 128/2	2	2	8	64	4.75	18	8
Non-extended spectrum cephalosporins (1st generation and 2nd generation cephalosporins)										
CEP	0.5-1024	≤ 2[a]	n/a	8	8	16	512	16.9	49	51
CEF (n=614)	0.5-1024	≤ 4	n/a	2	2	4	128	4.63	N/A	N/A
Extended-spectrum cephalosporins (3rd generation and 4th generation cephalosporins)										
CAZ	0.06-128	≤ 8	≥ 32	0.25	0.12	0.25	2	0.3	6	3
CPD	0.06-256	≤ 2	≥ 8	0.5	0.5	1	32	1.1	8	12[c]
CTX	0.06-64	≤ 8	≥ 64	≤ 0.06	≤ 0.06	1	4	0.3	7	3
Cephamycin										
FOX	0.5-1024	≤ 8	≥ 32	2	2	4	16	3.7	13	9
Carbapenem										
MEM	0.06-15	≤ 4	≥ 16	≤ 0.03	≤ 0.03	≤ 0.25	≤ 0.5	0.07	1	1
Tetracyclines										
DOX	0.25-128	≤ 0.12	≥ 0.5	2	2	2	16	1.9	12	9
Phenicols										
CHL	0.5-512	≤ 8	≥ 32	4	8	8	8	6.7	57[b]	6
Fluoroquinolones										
CIP (n=2939)	0.008-64	≤ 1	≥ 4	0.015	0.015	0.03	0.5	0.04	1	9
ENR	0.008-128	≤ 0.5	≥ 4	0.03	0.03	0.06	1	0.06	3	11[c]
Aminoglycosides										
GEN	0.25-128	≤ 4	≥16	0.5	0.5	1	2	0.75	7	5
AMK	0.12-128	≤ 16	≥ 32	2	2	4	8	1.39	0.4	n/a
Sulfonamides										
SXT	0.015-128	≤ 2	≥ 8	≤ 0.06	≤ 0.06	≤ 0.06	0.25	0.09	1	8

Table 2.1 MICs (µg/ml) statistics for all *E. coli* isolates and each of 17 antimicrobial agents.

[a] Breakpoint MICs were valid for urinary tract infections only. [b] *P* value (determined by Chi-square test) are shown where $P < 0.01$.

[c] *P* value (determined by Chi-square test) are shown where $P < 0.05$.

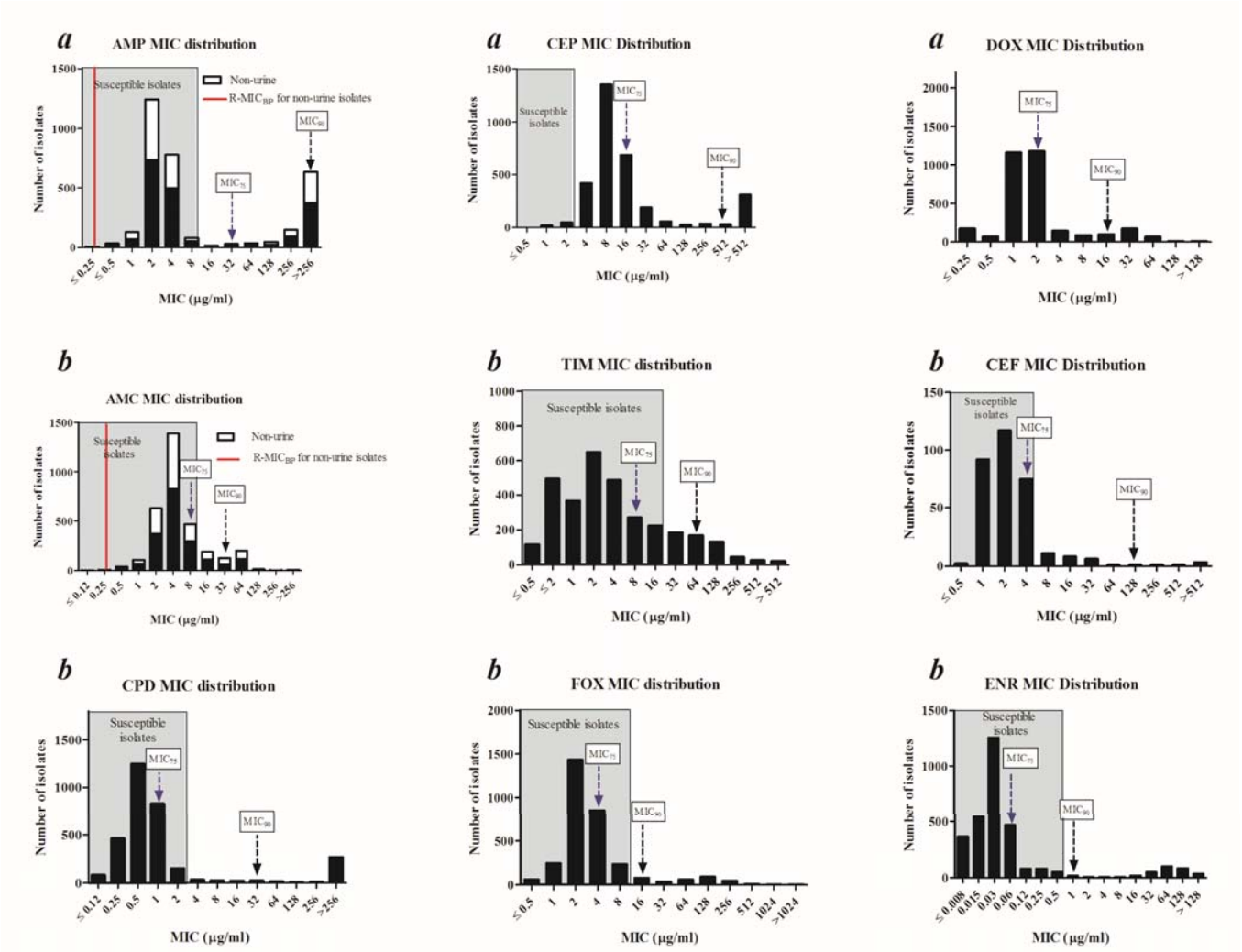

Figure 2.4 Population distribution of selected antimicrobials. The Y axis represents the numbers of isolates for each MIC (X axis). a) The MIC population was bimodal, with both MIC_{75} and MIC_{90} exceeding the susceptible MIC breakpoint ($S\text{-}MIC_{BP}$). b) Bimodal population distribution showed with the second mode and the MIC_{90} exceeding but the MIC_{75} below the $S\text{-}MIC_{BP}$

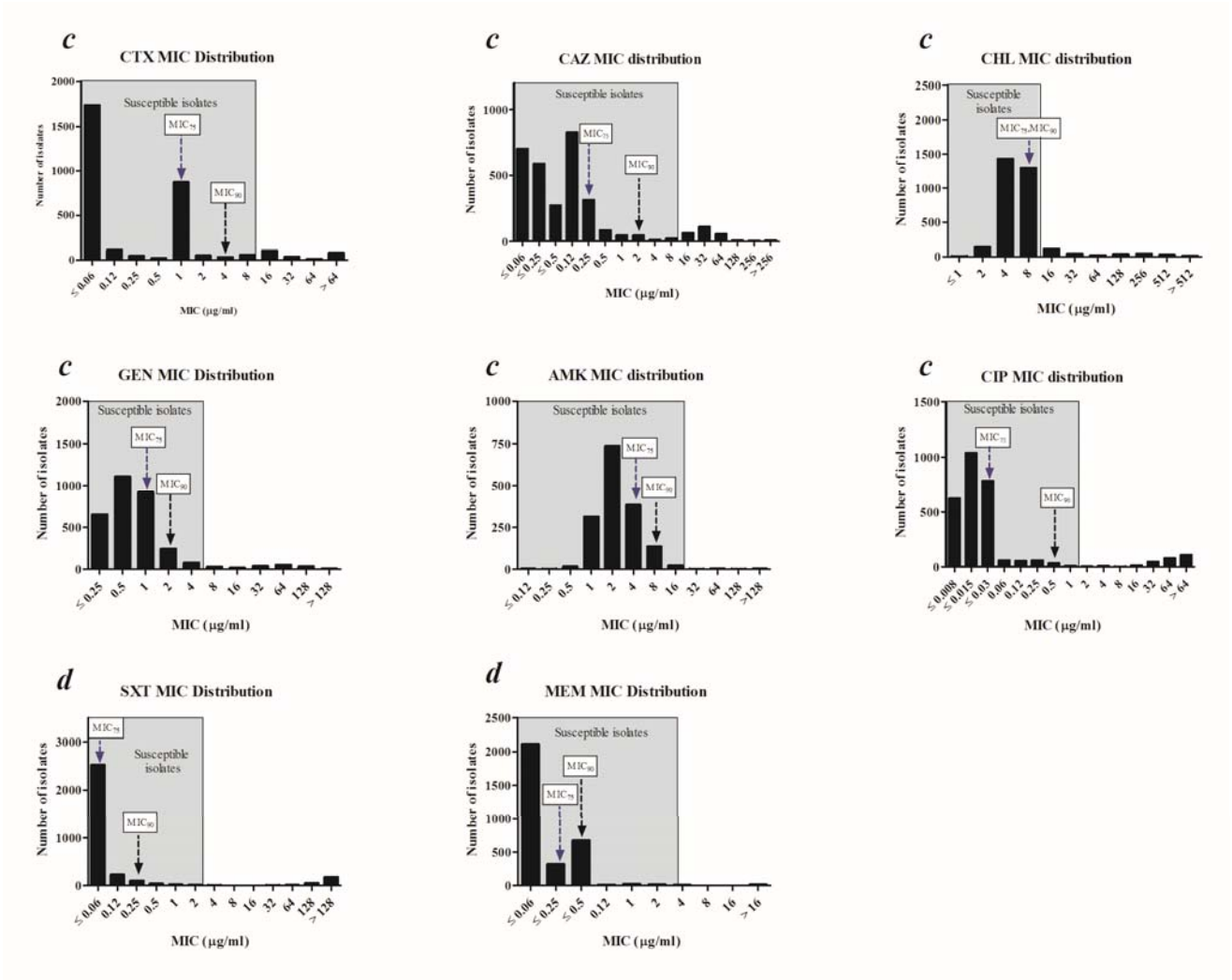

Figure 2.4 (cont.) Population distribution of selected antimicrobials. c) Drugs for which S-MIC$_{BP}$ were 1-2 fold higher than MIC$_{90}$ or at MIC$_{90}$ d) Drugs for which S-MIC$_{BP}$ were >2-fold higher than both the MIC$_{75}$ and MIC$_{90}$.

2.3.3 Percentages of Antimicrobial Resistance

The percent of isolates designated as resistant (i.e., either R or I as defined by CLSI) to at least one of the seventeen antimicrobials was summarized in Table 2.1. All isolates expressed resistance to at least one drug (i.e., NDR=0%). Order of the prevalence of percent resistance was DOX (100%), CEP (97.8%) > AMP (48.4%) > AMC (40.1%) > TIM (18.0%) > CPD (12.8%), FOX (10.9%), CEF (10.6%), CHL (9.6%) > ENR (10.4%), CIP (9.2%), CEF (8.1%), CAZ (8.0%), SXT (7.9%) > GEN (5.5%) > AMK(0.7%), MEM (0.6%) ($P< 0.0001$) (Table 2.2).

Percentages of resistance were greater in dogs compared to cats for all drugs ($P<0.05$), except for DOX, CEP and MEM, which were not different between dogs and cats ($P=0.47$ with power = 0.99). For dogs, the overall proportion of resistance was higher in males compared to females (Table 2.2), but there was no difference between male and female cats. However, differences in the proportion of resistance to each drug between males and females could be detected only for AMP and AMC (P=0.03). When considered by region, DOX (100%) and CEP (98.9%) had the highest percent of resistance for all regions; a significant difference could not be detected among the six regions ($P=0.38$ with power = 0.99). The percent of resistance of AMP and AMC were higher in SC region compared to the other regions (72.3%, 63.8%, respectively) (Table 2.2).

Drugs	Overall	Regions						Species		Gender	
		NC (n=466)	NE (n=714)	NW (n=107)	SC (n=179)	SE (n=441)	SW (n=774)	Canine (n=2390)	Feline (n=780)	Female (n=1869)	Male (n=782)
CEP	97.8 (97.3-98.3)[a]	97.0 (95.5-98.6)	98.0 (97.0-99.1)	99.1 (97.2-100)	98.9 (97.3-100)	98.4 (97.3-99.6)	98.2 (97.3-99.1)	98.0 (97.5-98.6)	97.2 (96.0-98.3)	97.9 (97.3-98.6)	98.5 (97.6-99.3)
AMP	48.4 (46.7-50.2)[a]	41.0 (36.5-45.5)	43.8 (40.2-47.5)	55.1 (45.7-64.6)	69.3 (62.5-76.0)	45.6 (40.9-50.2)	51.3 (47.8-54.8)	52.7 (50.7-54.7)	35.3 (31.9-38.6)	42.7 (40.5-44.9)	60.1[π] (56.7-63.5)
AMC	40.0 (38.3-41.7)[a]	32.6 (28.4-36.9)	32.4 (28.9-35.8)	50.5 (41.0-59.9)	64.3 (57.2-71.3)	36.3 (31.8-40.8)	45.0 (41.5-48.5)	45.2 (43.2-47.2)	24.2 (21.2-27.2)	33.8 (31.7-36.0)	52.9[π] (49.4-56.4)
TIM	18.0 (16.6-19.4)[a]	18.2 (14.7-21.8)	18.1 (15.3-20.9)	17.8 (10.5-25.0)	20.7 (14.7-26.6)	20.9 (17.1-24.7)	15.4 (12.8-17.9)	19.5 (17.9-21.0)	13.3 (11.0-15.7)	17.9 (16.2-19.7)	18.5 (15.8-21.3)
DOX	100 (99.9-100)	100 (99.2-100)	100 (99.5-100)	100 (96.6-100)	100 (98.0-100)	100 (99.2-100)	100 (99.5-100)	100 (99.9-100)	100 (99.5-100)	100 (99.8-100)	100 (99.5-100)
CPD	12.8 (11.6-14.0)[c]	12.5 (9.5-15.4)	10.8 (8.6-13.3)	13.1 (7.3-21.0)	16.2 (11.2-22.4)	15.4 (12.1-18.8)	11.0 (8.8-13.2)	13.9 (12.5-15.3)	9.5 (7.5-11.8)	11.8 (10.3-13.2)	14.2 (11.8-16.6)
FOX	10.9 (9.8-12.0)[d]	10.3 (7.5-13.1)	9.7 (7.5-11.8)	10.3 (4.5-16.0)	13.4 (8.4-18.4)	15.2 (11.8-18.5)	8.7 (6.7-10.6)	12.3 (11.0-13.6)	6.7 (4.9-8.4)	10.2 (8.9-11.6)	12.0 (9.7-14.3)
ENR	10.3 (9.3-11.4)[e]	12.7 (9.6-15.7)	8.7 (6.6-10.8)	6.5 (1.9-11.2)	13.4 (8.4-18.4)	12.2 (9.2-15.3)	9.3 (7.3-11.4)	11.7 (10.4-13.0)	5.9 (4.2-7.6)	10.1 (8.8-11.5)	11.0 (8.8-13.2)
CHL	9.6 (8.6-10.6)[f]	9.0 (6.4-11.6)	9.1 (7.0-11.2)	12.2 (6.0-18.3)	14.0 (8.9-19.0)	10.0 (7.2-12.8)	9.4 (7.4-11.5)	10.8 (9.6-12.0)	6.0 (4.4-7.7)	9.5 (8.2-10.9)	10.6 (8.5-12.8)
CIP	9.2 (8.1-10.2)[f]	10.4 (7.6-13.3)	7.4 (5.4-9.3)	4.1 (0.2-8.0)	13.1 (7.7-18.4)	11.2 (8.1-14.3)	7.1 (5.2-9.0)	10.4 (9.1-11.7)	5.3 (3.7-7.0)	8.1 (6.8-9.4)	9.8 (7.6-11.9)
CEF	8.9 (3.4-15.7)				N/A				N/A		N/A
CAZ	8.0 (7.1-9.0)[f]	7.9 (5.5-10.4)	6.9 (5.0-8.7)	8.4 (3.2-13.7)	11.7 (7.0-16.5)	10.9 (8.0-13.8)	5.9 (4.3-7.6)	9.4 (8.2-10.6)	3.9 (2.5-5.2)	7.2 (6.0-8.3)	9.9 (7.8-11.9)
SXT	7.9 (6.9-8.8)[f]	9.0 (6.4-11.6)	7.1 (5.3-9.0)	7.5 (2.5-12.5)	10.1 (5.7-14.5)	9.8 (7.0-12.5)	6.6 (4.8-8.3)	9.0 (7.9-10.1)	4.2 (2.8-5.6)	7.7 (6.5-8.9)	8.2 (6.3-10.1)
CTX	7.4 (6.5-8.4)	8.6 (6.0-11.1)	6.7 (4.9-8.6)	6.5 (1.9-11.2)	10.6 (6.1-15.1)	8.8 (6.2-11.5)	5.8 (4.2-7.5)	8.7 (7.5-9.8)	3.7 (2.4-5.1)	6.9 (5.8-8.1)	9.0 (7.0-11.0)
GEN	5.5 (4.7-6.2)	5.8 (3.7-7.9)	4.9 (3.3-6.5)	3.7 (0.1-7.3)	8.9 (4.8-13.1)	6.8 (4.5-9.2)	4.9 (3.4-6.4)	6.3 (5.3-7.3)	2.8 (1.7-4.0)	5.5 (4.4-6.5)	5.9 (4.2-7.5)
AMK	0.6 (0.1-1.2)	0.9 (0.2-1.2)	1.1 (0.7-1.5)	0	0	0	1.3 (0.4-2.0)	0.7 (0.1-1.6)	0.8 (0.3-1.1)	0.9 (0.4-1.8)	0.4 (0.1-0.7)
MEM	0.6 (0.4-0.9)[h]	1.1 (0.1-2.0)	0.6 (0.01-1.1)	0	0	0.2 (0-0.7)	1.2 (0.4-1.9)	0.5 (0.3-0.8)	0.9 (0.2-1.6)	0.8 (0.4-1.2)	0.5 (0.01-1.0)

Table 2.2 Percent of antimicrobial resistance by geographical region and demographic data for each of 17 antimicrobials.

[a] Percentages were significantly higher than other antimicrobials with $P < 0.05$. [b] Percentages were significantly ($P < 0.05$) higher when compare to other drugs except for CPD, [c] ENR, FOX, [d] CIP, CHL, ENR. [e] Percentages were significantly ($P < 0.05$) higher when compare to CTX, GEN, and MEM. [f] Percentages were significantly ($P < 0.05$) higher when compare to only GEN and MEM. [h] Percentage was least to other antimicrobials ($P < 0.05$).

To identify factors associated with antimicrobial R%, a multinomial logistic regression model was conducted using demographic data (e.g. species, gender, and age), collection site, and geographic region (Tables 2.4). Since R% of AMK and MEM were too low to analyze, this study could not identify risk factors of AMK and MEM resistance. Likewise, because all isolates were resistant to DOX, risk factors could not be assessed. Neither gender was associated with R% for any antimicrobials. The southern regions were risk factors for R%: the SC region for AMP, AMC, and GEN, the SE region for FOX, CIP and CAZ, and the SW region for TIM and CAZ. In contrast, the NE region was a risk factor for AMC ($P<0.05$). Species was a risk factor for most antimicrobials; the exceptions being CEP and CAZ ($P<0.05$). As such, canine isolates were more likely to develop resistance than feline isolates. Isolates from geriatric animals (12.5- $\geq$15) of both species were more likely to develop antimicrobial resistance for AMP ($P=0.01$), TIM ($P=0.01$), DOX ($P=0.007$), CPD ($P=0.03$), ENR ($P<0.0001$), CIP ($P<0.0001$), CAZ ($P=0.01$) and CTX ($P=0.01$) (Table 4). Age at 0-1 and 3-5 years were a risk factor for resistance to ENR ($P=0.04$) and CIP ($P=0.04$), and TIM ($P=0.02$), CPD ($P=0.005$), FOX ($P=0.02$), CAZ ($P=0.02$), respectively, when compared to age at 1-3 years. R% of different non-urinary sites were summarized in Table 2.3. Since the majority of *E. coli* isolates were collected from urine, the numbers for other collection sites were limited for analysis. Therefore, to identify whether or not location of infection was a risk factor of resistance, infection locations were classified as to either urine or non-urine. Isolates from non-urine were highly associated to R% of AMP (OR=52.1), AMC (OR=85.9), CPD (OR=1.4), and ENR (OR=1.3) (Table 2.4).

Drugs	Urine (n=1944)	Non-Urine										
		Ears (n=195)	Genital (n=92)	Skin (n=89)	Abscess (n=71)	Upper respiratory (n=49)	Wound (n=33)	Lung (n=30)	Body fluid (n=29)	Uterus (n=27)	Oral cavity (n=21)	Abdomen (n=17)
CEP	97.9 (97.3-98.6)	97.4 (95.2-99.7)	98.9 (96.7-100)	98.9 (96.6-100)	95.8 (88.1-99.1)	100 (92.8-100)	97.0 (84.2-99.9)	100 (88.4-100)	100 (88.1-100)	100 (87.2-100)	100 (83.9-100)	100 (80.5-100)
AMP	28.3 (26.3-30.4)	97.9 (95.9-99.9)	97.8 (94.7-100)	97.7 (94.6-100)	95.8 (88.1-99.1)	100 (92.8-100)	97.0 (84.2-99.9)	96.7 (82.8-99.9)	96.6 (82.2-99.9)	96.3 (81.0-99.9)	100 (83.9-100)	100 (80.5-100)
AMC	16.9 (15.2-18.5)	97.9 (95.9-99.9)	94.4 (89.7-99.2)	95.4 (91.0-99.8)	97.2 (90.2-99.7)	100 (92.8-100)	97.0 (84.2-99.9)	90.0 (73.5-97.9)	100 (88.1-100)	92.6 (75.7-99.1)	95.2 (76.2-99.9)	100 (80.5-100)
TIM	17.6 (15.9-19.4)	12.4 (7.8-17.1)	18.9 (10.8-27.0)	16.1 (8.4-23.8)	21.1 (12.3-32.4)	24.5 (13.3-38.9)	27.3 (13.3-45.5)	23.3 (9.9-42.3)	37.9 (20.7-57.7)	3.7 (0.09-19.0)	23.8 (8.2-47.2)	29.4 (10.3-56.0)
DOX	100 (99.8-100)	100 (98.1-100)	100 (96.0-100)	100 (95.9-100)	100 (94.9-100)	100 (92.8-100)	100 (89.4-100)	100 (88.4-100)	100 (88.1-100)	100 (87.2-100)	100 (83.9-100)	100 (80.5-100)
CPD	11.5 (10.0-12.9)	10.4 (6.1-14.7)	13.3 (6.3-20.4)	18.4 (10.3-26.5)	15.5 (8.0-26.3)	18.4 (8.8-32.0)	24.2 (11.1-42.3)	13.3 (3.8-30.7)	20.7 (8.0-39.7)	7.4 (0.9-24.3)	14.3 (3.1-36.3)	23.5 (6.8-49.9)
FOX	10.1 (8.7-11.4)	8.3 (4.4-12.2)	11.1 (4.6-17.6)	12.6 (5.7-19.6)	14.1 (6.0-24.4)	14.3 (5.9-27.2)	24.2 (11.1-42.3)	13.3 (3.8-30.7)	24.1 (10.3-43.5)	7.4 (0.9-24.3)	19.1 (5.5-41.9)	11.8 (1.5-36.4)
ENR	9.8 (8.5-11.1)	9.3 (5.2-13.4)	3.3 (0-7.0)	11.5 (4.8-18.2)	11.3 (5.0-21.0)	16.3 (7.3-29.7)	21.2 (9.0-38.9)	30.0 (14.7-49.4)	20.7 (8.0-39.7)	7.4 (0.9-24.3)	14.3 (3.1-36.3)	23.5 (6.8-49.9)
CHL	9.5 (8.2-10.8)	9.3 (5.2-13.4)	4.4 (0.2-8.7)	14.9 (7.5-22.4)	8.5 (3.2-17.5)	18.4 (8.8-32.0)	12.1 (3.4-28.2)	16.7 (5.6-34.7)	20.7 (8.0-39.7)	18.5 (6.3-38.1)	4.8 (0.1-23.8)	11.8 (1.5-36.4)
CEF	9.1 (2.3-17.1)						8.2 (3.4-14.4)					
CIP	8.0 (6.7-9.4)	8.7 (4.6-12.8)	3.6 (0-7.6)	12.4 (5.2-19.5)	7.5 (2.5-16.7)	8.5 (2.4-20.4)	21.4 (8.3-40.1)	27.6 (12.7-47.2)	25.0 (9.8-46.7)	4.0 (0.1-20.4)	14.3 (3.1-36.3)	25.0 (7.3-52.4)
CAZ	7.3 (6.1-8.4)	4.7 (1.7-7.6)	6.7 (1.5-11.8)	9.2 (3.1-15.3)	12.3 (6.0-22.7)	14.3 (5.9-27.2)	21.2 (9.0-38.9)	10.0 (2.1-26.5)	20.7 (8.0-39.7)	0	9.5 (1.2-30.4)	11.8 (1.5-36.4)
SXT	7.4 (6.2-8.6)	5.2 (2.1-8.3)	7.8 (2.2-13.3)	9.2 (3.1-15.3)	5.6 (1.6-13.8)	16.3 (7.3-29.7)	18.2 (7.0-35.5)	16.7 (5.6-34.7)	20.7 (8.0-39.7)	0	19.1 (5.5-41.9)	23.5 (6.8-49.9)
CTX	6.8 (5.7-7.9)	5.2 (2.1-8.3)	5.6 (0.8-10.3)	10.3 (4.0-16.7)	7.0 (2.3-15.7)	14.3 (5.9-27.2)	21.2 (9.0-38.9)	10.0 (2.1-26.5)	20.7 (8.0-39.7)	0	9.5 (1.2-30.4)	11.8 (1.5-36.4)
GEN	5.1 (4.1-6.1)	3.6 (1.0-6.3)	5.6 (0.8-10.3)	4.6 (0.2-9.0)	2.8 (0.3-9.8)	14.3 (5.9-27.2)	21.2 (9.0-38.9)	23.3 (9.9-42.3)	6.9 (0.09-22.8)	0	14.3 (3.1-36.3)	17.7 (3.8-43.4)
AMK	0.9 (0.3-1.4)	0.1 (0-0.4)	0.3 (0-0.5)	0.2 (0-0.5)	0	3.2 (1.8-4.2)	2.4 (1.3-2.9)	0	0	0	N/A	0
MEM	0.8 (0.4-1.3)	0	0	1.2 (0-3.4)	0	2.0 (0.05-10.9)	0	3.3 (0.08-17.2)	0	0	0	0

Table 2.3 Percent of antimicrobial resistance by infection location for each of 15 antimicrobials.

Risk Factors		AMP	CEF	AMC	TIM	DOX	CPD	FOX	ENR	CHL	CIP[a]	CAZ	SXT	CTX	GEN
Region	NE	0.9 (0.7-1.2)	1.6 (0.7-3.4)	0.7 (0.5-0.9)[b]	1.0 (0.7-1.3)	n/a	0.9 (0.6-1.3)	1.0 (0.7-1.5)	0.6 (0.4-0.9)	1.1 (0.7-1.7)	0.6 (0.4-1.0)	0.9 (0.6-1.4)	0.7 (0.5-1.1)	0.8 (0.5-1.3)	0.9 (0.5-1.5)
	NW	0.9 (0.5-1.6)	3.2 (0.4-24.9)	1.1 (0.6-2.0)	0.9 (0.5-1.6)	n/a	0.9 (0.5-1.8)	0.9 (0.4-1.9)	0.4 (0.2-1.0)	1.5 (0.8-2.9)	0.3 (0.1-0.9)	0.9 (0.4-2.0)	0.7 (0.3-1.6)	0.6 (0.2-1.5)	0.5 (0.1-1.6)
	SC	1.5 (0.9-2.4)[a]	2.6 (0.6-11.9)	1.6 (0.9-2.7)[a]	1.2 (0.7-1.8)	n/a	1.3 (0.8-2.2)	1.4 (0.8-2.4)	1.0 (0.6-1.6)	1.8 (1.0-3.1)	1.1 (0.6-2.0)	1.5 (0.9-2.8)	1.0 (0.5-1.9)	1.2 (0.7-2.3)	1.6 (0.8-3.2)[a]
	SE	1.1 (0.8-1.5)	1.8 (0.7-4.6)	1.1 (0.8-1.6)	1.2 (0.9-1.7)	n/a	1.3 (0.9-1.9)	1.7 (1.1-2.5)[b]	0.9 (0.6-1.4)	1.2 (0.8-1.9)	1.0 (0.7-1.6)[a]	1.5 (0.9-2.3)[a]	1.1 (0.7-1.8)	1.1 (0.7-1.8)	1.3 (0.7-2.2)
	SW	0.9 (0.7-1.2)	1.8 (0.8-3.9)	0.9 (0.6-1.3)	0.8 (0.6-1.1)[a]	n/a	0.9 (0.6-1.3)	0.9 (0.6-1.3)	0.7 (0.4-1.0)	1.1 (0.8-1.7)	0.6 (0.4-1.0)	0.7 (0.5-1.2)[a]	0.7 (0.4-1.1)	0.7 (0.4-1.0)	0.9 (0.5-1.5)
	NC	1 (reference)													
Species	Canine	1.4 (1.1-1.8)[a]	1.8 (1.0-3.6)	1.6 (1.2-2.2)[b]	1.7 (1.3-2.3)[b]	n/a	1.6 (1.1-2.2)[a]	1.9 (1.3-2.7)[b]	3.1 (2.0-4.7)[c]	2.1 (1.4-3.1)[b]	3.6 (2.2-5.9)[c]	2.7 (1.7-4.4)[c]	2.3 (1.4-3.6)[b]	2.6 (1.6-4.2)[b]	2.4 (1.4-4.3)[b]
	Feline	1 (reference)													
Gender	Female	0.9 (0.7-1.1)	0.8 (0.4-1.6)	0.9 (0.7-1.2)	1.0 (0.8-1.2)	n/a	0.8 (0.7-1.1)	0.9 (0.7-1.2)	0.9 (0.7-1.2)	0.9 (0.7-1.2)	0.8 (0.6-1.2)	0.7 (0.5-1.0)	1.0 (0.7-1.4)	0.8 (0.6-1.1)	1.1 (0.7-1.6)
	Male	1 (reference)													
Age	0-1	0.8 (0.5-1.3)	0.9 (0.3-3.1)	1.1 (0.6-2.1)	0.9 (0.6-1.6)	n/a	1.0 (0.6-1.7)	0.8 (0.5-1.5)	1.0 (0.5-2.0)[a]	1.1 (0.6-2.1)	0.8 (0.4-2.0)[a]	1.2 (0.7-2.4)	1.0 (0.4-2.1)	1.7 (0.8-3.5)	1.0 (0.4-2.2)
	3.1-5	0.9 (0.6-1.6)	1.1 (0.3-4.0)	1.3 (0.7-2.3)	0.8 (0.4-1.3)[a]	n/a	0.5 (0.3-0.9)[b]	0.5 (0.2-0.9)[a]	1.2 (0.6-2.5)	0.8 (0.4-1.5)	1.0 (0.5-2.2)	0.6 (0.3-1.4)[a]	1.1 (0.5-2.4)	0.8 (0.3-1.8)	0.9 (0.4-2.1)
	5.1-7.5	1.0 (0.6-1.6)	1.7 (0.5-6.5)	1.1 (0.6-2.0)	1.1 (0.7-1.8)	n/a	0.8 (0.5-1.4)	0.7 (0.4-1.3)	1.2 (0.6-2.4)	0.8 (0.4-1.4)	1.1 (0.5-2.4)	1.0 (0.5-2.1)	1.5 (0.8-3.1)	0.9 (0.4-2.0)	0.8 (0.3-1.7)
	7.6-10	0.9 (0.6-1.4)	2.6 (0.7-9.1)	1.5 (0.9-2.4)	1.3 (0.8-2.0)	n/a	1.1 (0.7-1.8)	1.0 (0.7-1.7)[a]	1.6 (0.9-3.0)	1.1 (0.7-1.9)	1.5 (0.7-2.9)	1.6 (0.9-2.9)	1.1 (0.6-2.2)	1.7 (0.9-3.1)	1.2 (0.6-2.4)
	10.1-12.5	0.9 (0.6-1.4)	1.5 (0.5-4.5)	0.9 (0.5-1.5)[a]	1.1 (0.7-1.7)	n/a	0.8 (0.5-1.3)	0.6 (0.4-1.0)	1.8 (1.0-3.3)	1.1 (0.7-1.9)	1.8 (0.9-3.5)	1.1 (0.6-2.1)	1.6 (0.9-3.1)	1.4 (0.7-2.6)	1.0 (0.5-2.0)
	12.6-15	1.1 (0.7-1.7)	1.2 (0.4-3.7)	1.5 (0.9-2.6)	1.4 (0.9-2.3)	n/a	1.1 (0.7-1.9)	1.0 (0.6-1.7)	2.3 (1.2-4.4)[a]	1.1 (0.6-2.0)	2.2 (1.1-4.4)[a]	1.4 (0.7-2.7)	1.7 (0.8-3.4)	1.2 (0.6-2.9)	1.2 (0.5-2.5)
	>15	1.5 (0.9-2.4)[a]	1.6 (0.4-5.8)	1.6 (0.9-2.9)	1.8 (1.0-3.0)[a]	n/a	1.5 (0.8-2.6)[a]	1.0 (0.5-1.9)	4.1 (2.0-8.3)[c]	1.1 (0.5-2.3)	4.2 (1.9-9.2)[c]	2.2 (1.0-4.9)[a]	2.0 (0.9-4.4)	2.4 (1.1-5.4)[a]	1.5 (0.6-3.7)
	1.1-3	1 (reference)													
Infection location	Non-urine	52.1 (36.1-75.1)[c]	1.0 (0.5-2.1)	85.9 (60.2-123)[c]	1.1 (0.9-1.4)	n/a	1.4 (1.0-1.8)[a]	1.2 (0.9-1.7)	1.3 (1.0-1.8)[b]	1.0 (0.7-1.3)	1.4 (1.0-2.0)	1.3 (0.9-1.8)	1.3 (1.0-1.9)	1.3 (0.9-1.9)	1.3 (0.9-1.9)
	Urine	1 (reference)													

Table 2.4 Odds ratios for the association of percent of antimicrobial resistance for each of 15 antimicrobials and geographical region.

[a] *P<0.05*, [b] *P<0.01*, [c]*P<0.0001.*

2.3.4 Type of Resistance

Based on 12 antimicrobial categories, the proportion of MDR isolates (52%) was greater than SDR (46%) which was greater than XDR (2%; *P<0.0001*). No isolate expressed NDR or PDR phenotypes. When considered among the 12 antimicrobial categories, CEP, and CHL resistances were highly associated with MDR, with odds ratios (OR) >999.9 (95% CI 367.8-999.9; *P<0.0001*), and 3.45 (95% CI 1.4-9.0; *P=0.001*, respectively with power (or β=0.91). In contrast, AMP, AMC, FOX, CPD, GEN, TIM and SXT were least associated with MDR (*P<0.001*). Of the 1647 SDR isolates, DOX or CEP resistance (100% and 99%, respectively) were the most commonly expressed phenotypes; this group included only CEP and DOX resistance (81.8%) or CEP and DOX with another antimicrobial category (17.4%). The 5 least common antimicrobial categories to which these isolates expressed resistance were cephamycins (0.2%), extended-cephalosporin (0.3%), aminoglycosides (0.5%), anti-pseudomonal penicillins with β-lactamase inhibitors (0.5%), and fluoroquinolones (1.0%).

Risk factors associated with SDR, MDR, and XDR isolates were summarized in Table 2.5. No association could be detected among types of resistance with gender (*P>0.05*). Based on location of infection (Figure 2.3), the proportion of MDR from non-urinary tract (92.8%; 739/796) was higher than that from urinary tract (23.7%; 454/1913; *P<0.0001*) with OR 47.1 (*P<0.0001*). In contrast, the proportion of SDR isolates from the urinary tract (74.0%; 1416/1913) was greater than that from of the non-urinary tract isolates (5.9%; 47/796; *P<0.0001*) with OR 0.02 (*P<0.0001*). XDR isolates were associated with non-urinary isolates and isolates collected from dogs compared to cats with OR=35.4 and 3.0, respectively (*P<0.01* and *=0.01*). The proportion of MDR was higher than SDR for non-urinary tract isolates

(*P<0.0001*). The proportion of isolates from the ears expressing MDR was 93%, 2% of XDR, and 5% of SDR.

Risk factors		N	SDR		MDR		XDR	
			OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**
Region	NE	715	1.0 (0.7-1.3)	0.10	1.0 (0.8-1.3)	0.08	0.6 (0.2-1.3)	0.97
	NW	107	0.6 (0.4-1.1)	0.19	1.6 (0.9-2.7)	0.20	N/A	0.96
	SC	179	0.5 (0.4-0.9)[a]	0.03	1.8 (1.1-3.0)	0.02	1.3 (0.4-4.0)	0.96
	SE	442	0.9 (0.7-1.3)	0.39	1.1 (0.8-1.5)	0.53	1.8 (0.9-3.9)	0.95
	SW	777	1.1 (0.8-1.4)	0.01	0.9 (0.7-1.2)[a]	0.01	0.5 (0.2-1.1)	0.98
	NC	467	Reference					
Species	Canine	2037	0.6 (0.5-0.8)	0.69	1.6 (1.3-2.1)[b]	0.0001	3.0 (1.2-7.2)[a]	0.01
	Feline	664	Reference					
Gender	Female	1865	1.0 (0.8-1.2)	0.96	1.0 (0.8-1.3)	0.98	0.6 (0.4-1.1)	0.11
	Male	784	Reference					
Age	0-1	228	1.2 (0.7-2.0)	0.33	0.8 (0.5-1.3)	0.24	1.2 (0.3-5.9)	0.47
	3.1-5	241	1.1 (0.7-1.9)	0.73	0.9 (0.6-1.6)	0.86	1.4 (0.3-6.1)	0.57
	5.1-7.5	273	1.3 (0.8-2.0)	0.25	0.8 (0.5-1.3)	0.25	2.1 (0.5-8.2)	0.64
	7.6-10	517	1.1 (0.7-1.6)	0.94	1.0 (0.6-1.5)	0.92	1.9 (0.5-7.1)	0.74
	10.1-12.5	568	1.2 (0.8-1.8)	0.32	0.9 (0.6-1.3)	0.29	2.0 (0.6-7.5)	0.59
	12.6-15	378	1.0 (0.6-1.5)	0.57	1.1 (0.7-1.7)	0.46	2.0 (0.5-8.4)	0.71
	> 15	242	0.7 (0.4-1.2)[a]	0.01	1.4 (0.9-2.3)[a]	0.01	3.2 (0.6-16.3)	0.24
	1.1-3	206	Reference					
Infection location	Non-urine	796	0.02 (0.01-0.03)[c]	<0.0001	47.1 (31.4-70.8)[c]	<0.0001	35.4 (17.9-70.3)[c]	<0.0001
	Urine	1913	Reference					

Table 2.5 Odds ratio for the association of resistant types: non-MDR, MDR and PDR and region, demographical data and infection location.

[a] *P<0.05*, [b] *P<0.01*, [c]*P<0.0001.*

2.4 Discussion

This study investigated the patterns of antimicrobial resistance of clinical canine and feline *E. coli* and their relationships with other factors that might increase the risk of resistance. These factors included demographic data, signalment, and location of infection. This study was not able to discriminate between *E. coli* isolates that were colonizing the sampled site versus those that were pathogenic, i.e., associated with clinical signs of infection in those areas characterized by a commensal population (e.g. genital area, upper respiratory tract, or oral cavity). However, because the majority of isolates were collected from extraintestinal sites normally expected to be sterile (e.g. urine, lung or body fluid), such isolates were considered pathogenic. Therefore, *E. coli* isolates of this study were ExPEC.

The ExPEC collected from dogs and cats in this study had similar locations as described in both humans [21] [22] [23] and dogs [24] [25]. The high proportion of isolates collected from the urine may reflect, in part, the frequency of UTIs in females, but also ease of sample collection.

In this study, a high R% of β-lactam resistance was documented by AMC, AMP, and CEP with 40, 48, and 98% of isolates expressing resistance to each drug, respectively (Table 2.2). The resistance to CEP and DOX (100%) was not expected nor has it been previously reported. However, it likely reflects, in part, a change, and specifically a lower S-MIC_{BP} recently delineated by CLSI VET01-4 [12]. Previously, CLSI indicated the S-MIC_{BP} of CEP and DOX to be 8 and 4 μg/ml, respectively [12], but it has now decreased it to < 2 and < 0.12 μg/ml, respectively [12]. S-MIC_{BP} of AMP and AMC also have been decreased in recent years such that non-urinary isolates are considered susceptible to either drug only with an MIC ≤ 0.25 μg/ml. However, for urine isolates, a higher breakpoint has been set at ≤ 8, presumably reflecting

anticipated higher concentrations in urine for both of these excreted drugs via kidney (Figure 2.2). Previous CLSI standard have stated that the S-MIC_{BP} would be weighted towards microbial population distributions rather than towards clinical outcomes in relation to MIC [12]. The recent CLSI standard, however, indicate that the purpose of the clinical S-MIC_{BP} is to guide the selection of drugs based on the likelihood of achieving effective concentrations of drugs. As such, S-MIC_{BP} is specific to the disease-indication, infection location (for example urine vs non-urine, or soft tissue) and target-animal-species. The criteria are based on correlation of an effective concentration of drug exposure or/and an effective course of therapy [12]. Clinically, using the S-MIC_{BP} as a basis of drug selection, rather than a higher concentration that is yet below the R-MIC_{BP}, might facilitate avoidance of repeated exposure to otherwise inappropriate antimicrobials that are less likely to achieve effective concentrations at the site of infection. This study suggests that therapeutic success may be enhance that antimicrobial based on susceptibility testing rather than empirical selection. An additional concern raised by this study is the magnitude of MIC for isolates considered susceptible for selected drugs (e.g., AMX, AMC, and ENR). A large proportion of the susceptible isolates (in the grey box of Figure 4) have MIC that are much higher than wild-type isolates (that is, isolates expressing minimal resistance to the drug). The MIC are close to the S-MIC_{BP}, indicated that some levels of resistance have emerged but not enough for the isolate to be classified as resistant. Current commercial susceptibility testing does not test at concentrations low enough to detect MIC indicative of emerging resistance. As such, care should be taken to not over interpret an "S" designation on a culture report to mean that no resistance has emerged in the isolate.

The 100% of DOX resistance demonstrated in this study may not reflect the acquired resistance [12]. Rather, this study suggests that DOX is ineffective for *E. coli* infection, that is, *E.*

coli might be inherently resistant to DOX. As such, DOX probably should not be recommended for routine uses of *E. coli* Infection. This is particular true for urinary tract infections since much of DOX is excreted through the intestinal tract, and as such, urinary concentrations might be low, as is suggested by the Urinary Guidelines [26].

Surprisingly, the R% of AMP did not significantly differ from AMC (power 0.99), indicating that the addition of a β-lactamase inhibitor (clavulanic acid) would not be expected to increase the efficacy of aminopenicillins against *E. coli* infection. This finding supports the recommendation provided in the antimicrobial use guidelines for treatment of UTI in dogs and cats which list amoxicillin (modeled by AMP) as a first choice drug [26]. The R% of AMP of this study was similar to studies of *E. coli* isolates from human medicine. Resistance to AMP in human uropathogenic *E. coli* has substantially increased over time, being 37% in isolates collected from 1995-2001 [21] and 52.8% in isolates collected during 2004-2006 [27]. More recently, Sanchez et al. [28] described increasing R% of AMP (range of 37-41%) across time from year 2000 to 2010 in adult outpatients. In contrast to AMP, AMC resistance in this study (40%) was much higher than that in human medicine studies: 23.4% [27] and 3.7-8.6% [28]. Further, in this study, the R% for AMC (40%) was higher than expected. Resistance to AMC generally reflects β-lactamase production as was demonstrated in a study of UPEC from Portugal. β-lactam resistance in either non-ESBL-producing SDR or MDR *E. coli* isolates is more likely to be mediated by non-transferable TEM-lactamase enzymes (TEM-1, TEM-2) carried by transposons, which are then integrated into the chromosomal DNA [29]. Another reason that R% to AMC may be higher in dogs compared to humans may be more common use of AMC in dogs and cats compared to humans. Our findings suggest that clinical use of β-lactam and β-lactamase inhibitor combinations in dogs and cats may contribute to a strongly selective

pressure to develop resistant bacterial isolates which were resistant to AMP, AMC and CEP. This is supported by other studies that have demonstrated emergence of resistance in faecal *E. coli* after treatment with therapeutic doses of selected antimicrobials in dogs [29]. Another reason that R% to AMC may be higher in dogs compared to humans may be more common use of AMC in dogs and cats compared to humans. The differential R% in animal vs people supported species specific surveillance.

In this study, as directed by CLSI, CEP and CEF were used as a model for 1st generation cephalosporins [12]. CEF is often used prophylactically for surgery in dogs or cats and as such, use is generally short term [29]. In contrast, cephalexin is the first drug choice for treatment of *Staphylococcal* skin infection in dogs [30] [31]. CEP had the second highest R% among 17 drugs with both MIC_{75} and MIC_{90} were100-fold higher than the S-MIC_{BP}. It is tempting to speculate that the reason for this markedly high proportion of resistance to CEP is the common use of cephalexin for treatment of pyoderma in dogs [31]. Results of this study indicate that CEP is not a wise treatment for *E. coli* infections. Of note, 2004 National Committee on Clinical Laboratory Standards state that CEP susceptibility predicts that of cephalexin and other cephalosporins but not CEF [32]. Further, a study of *E. coli* uropathogen in humans demonstrated that some CEF-susceptible *Enterobacteriaceae* isolates may be cephalexin-resistant [33]. Our findings also support different susceptibility patterns for CEF and CEP with R% of CEP being much higher than that of CEF. Consequently, CEP susceptibility results should not be used to interpret CEF.

In this study, the lowest prevalence of *E. coli* resistance was expressed toward MEM (0.6%), AMK (0.6%), and GEN (5.4%). For each drug, MIC_{90}, MIC_{75} and MIC_{50} were well below the MIC_{BP-S} (Table 2.1, Figure 2.4). Consistent with the presumed relationship between antimicrobial use and resistance, this low R% to each of these higher tier antimicrobials probably

reflects their limited use due to cost, inconvenient dosing interval (MEM), safety (AMK, and GEN), as well as their importance to human [34]. Another surprising finding was the relatively low R% for SXT (7.9%). This may reflect a general decline in the use of this drug over the past decade by veterinarians concerned about sulphonamides induced adverse effects [35]. It is important to note that sulfamethoxazole is the model sulphonamide drug for use in dogs and cats (and humans), yet no pharmacokinetic information is available for this sulphonamide in either species. Thus, the relevance of the breakpoint established by CLSI to dog and cat susceptibility testing is not clear.

As with other investigations, this study demonstrated a low prevalence of resistance (approximating 10%) to fluoroquinolones (including CIP and ENR), despite their popularity [36] [37] [38] [39] [40]. However, as with previous investigations, this study confirmed that ENR resistance was highly associated with MDR isolates (with OR = 1.4; Table 3 and Table 4; [36] [21]. In a previous publication, we reported that only 3% of the 84 *E. coli* isolates expressing single drug resistance were resistant to ENR, compared to 85% expressing SDR against β-lactams [41]. Also, 50% of the isolates had an MIC of 0.03 or more and MIC_{90} of ENR is above S-MIC_{BP}, which is indicative of first step mutation providing a minimum level of resistance to ENR (Liu et al., 2012). Since fluoroquinolone resistance usually occurs through stepwise accumulation of point mutations which affect the likelihood of selection of first-step resistant mutants [42] [43], ENR resistance in *E. coli* is commonly both high-level and associated with MDR. This relationship appears to reflect induction of efflux pumps whose substrates' specificity includes a variety of drugs [44] [43]. These findings suggest that despite the low prevalence of resistance, empirical first choice use of fluoroquinolones may not be prudent, as is suggested by the Urinary Guidelines [26].

Collection site was a risk factor for level of resistance. Isolates collected from the urinary tract were more commonly associated with SDR, whereas MDR or XDR were associated with non-urine sites (OR = 47.1; $P<0.0001$). This may reflect the tendency of isolates from non-urinary sites (e.g., ear, abscess, skin) to be more likely associated with co-infection or colonization with other bacteria. Such cohabitation may result in sharing of resistant genes whose expression may result in multiple mechanisms and thus MDR [45]. Canine vs feline isolates appear to be a risk factor associated with XDR ($P=0.01$) and MDR ($P=0.0001$) *E. coli* resistance. This may reflect more common use in dogs compare to cats. This findings suggest the need for investigating antimicrobial use patterns in dogs and cats.

2.5 Conclusions

This study demonstrates that no *E. coli* pathogens collected from dogs and cats express susceptible to at least one drug. Further, the percent of canine and feline *E. coli* pathogens resistant to AMC, AMP, CEP, and DOX is high. The MIC_{90} of these drugs exceeds the $R\text{-}MIC_{BP}$ by 8-fold indicating that when resistance occurs, it is high-level resistance. As such, empirical use of selected antimicrobials to treat *E. coli* infection in dogs and cats may be ill advised, indicating that culture and susceptibility testing may become more important, particularly in patients whose infections are complicated. Further, this data suggests that resistance may be greater in southern regions and in tissues other than the urine. Finally, this study supports the need for robust surveillance programs for antimicrobial use relating to antimicrobial resistance in dogs and cats.

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CHAPTER 3

ANTIMICROBIAL PRESCRIBING PRACTICES FOR TREATMENT OF *ESCHERICHIA COLI* INFECTIONS IN DOGS AND CATS IN THE UNITED STATES

3.1 Introduction

Emerging antimicrobial resistance is a major concern of healthcare in both human and veterinary medicine because of the therapeutic failure, increased patients morbidity and mortality, and health care costs. In general practices, some common infections are becoming increasingly difficult to treat and illness due to antimicrobial resistant bacteria may take longer to resolve [1].

Widespread antimicrobial use contributes to development of resistant populations which in turn can transfer resistance genes to susceptible populations [2]. To effectively prevent and control resistance, medical communities need to monitor and limit antimicrobial use [3]. Once a resistance strain has emerged, redeveloping susceptibility to antimicrobial therapy is a difficult and lengthy process [4]. Therefore, a proactive strategy should be focused on preventing the emergence of antimicrobial resistant strains through prudent use of antimicrobials, rather than trying to reduce the prevalence of resistant strains once they have emerged.

In human medicine, several approaches for controlling of antimicrobial use have been attempted. These include development of clinical practice guidelines, restriction of available antimicrobials, use of antimicrobial forms, feedback activities use of antimicrobial rotation programs, and requiring approval of use of certain drugs [5] [6] [7]. Comparatively, less strategies of antimicrobial use have been applied in companion animals. Detailed antimicrobial use data are required to better understand how antimicrobials are used in small animal veterinary

medicine, provide objective data for interpreting risks of antimicrobial resistance, and to establish a baseline from which to assess the effectiveness of measures taken to reduce antimicrobial use.

Monitoring and surveillance are an important part of any program because they provide feedback on the status of the program and allow antimicrobial use to be evaluated at various levels of veterinary professionals: within practices, regionally, and nationally [8]. These data should be reviewed and compared with antimicrobial use protocols [9] [10] to identify drug use that appears to be inconsistent or of concern.

Recently, a limited number of studies have documented the relationship between antimicrobial prescription patterns and prevalence of resistance in veterinary small animal hospitals [11], yet none have documented use in private veterinary hospitals in the United States. The goal of this study was to describe therapeutic antimicrobials prescribing behaviors of small animal practices in private veterinary hospitals in the United States, and to determine the prescribing behavior factors relative to antimicrobial resistance in *E. coli* infections. We hypothesized that the proportion of resistance can be correlated with veterinarian antimicrobial prescribing behaviors conducive to the emergence of resistance

3.2 Materials and methods

3.2.1 Sample collection and susceptibility testing

Isolates (n=3172) of clinical *E. coli* were from chapter 2.

3.2.2 Questionnaires

Veterinary clients submitting samples received introductory letters followed by a phone call during which information was collected using the questionnaire. Information collected

included demographic clinic data (zip, code, number of clinicians [1-5, 6-10, 11-15, 16-20, and >20], and specialties represented in the practice), patient signalment characteristics (patient species, breed gender, age, and neutered status), and information regarding the infection (patient factors [other illness, or prescriptions], history of infection [severity of infection, duration of episode infection, hospitalization]) and the therapeutic antimicrobial intervention, including, prescribing behaviors, and response of antimicrobial therapy (total response, persistent, and recurrent infections). Prescribing behaviors included identification of a drug of choice, and dosing dose regimens (onset of therapy [related to sample collection], route, dose, interval, and duration of treatment) [15].

3.2.3 Statistical analysis

The database from the surveys was recorded in Microsoft office access® 2007 software and transferred to spreadsheets (Microsoft office excel® 2013) for generating data. All data were manipulated and analyzed by using SAS software 9.3 (SAS Institute Inc., Cary, NC). Categorical variables were described using count and percentages with the relative 95% confidence interval (95% CI). The Mantel-Haenszel chi-square test [16] within PROC FREQ was used to compare the proportion of prescriptions between species, and location infections, success rate of antimicrobial therapy, and type of resistance.

The percentage of resistance of each antimicrobial and type of resistance were univariately compared for independent variables, such as number clinicians, age of patient, source of isolates, or gender, gender and number of microorganisms. Using multivariate, logistic regression, the relative risks for the dependent variable, types of resistance, were estimated for the independent variables to approach a generalized estimating equation model, allow to analysis

of correlated factors and calculate odd ratios (OR) [17]. All tests of significance were considered at $P<0.05$.

3.3 Results

3.3.1 Demographic data

A total of 3,172 *E. coli* isolates from different dogs and cats were submitted to PDL for susceptibility testing. Of these 823 patients were included in the survey through veterinary clinician response (response rate of 26%). These 823 *E. coli* isolates represented 552 small animal practices located in 6 different regions (Northwest, North-central, Northeast, Southwest, South-central and Southeast; Figure 3.1). Majority of *E. coli* isolates were from private clinics with 1-5 clinicians (74%), followed by 6-10 clinicians (19%) (Figure 3.1).

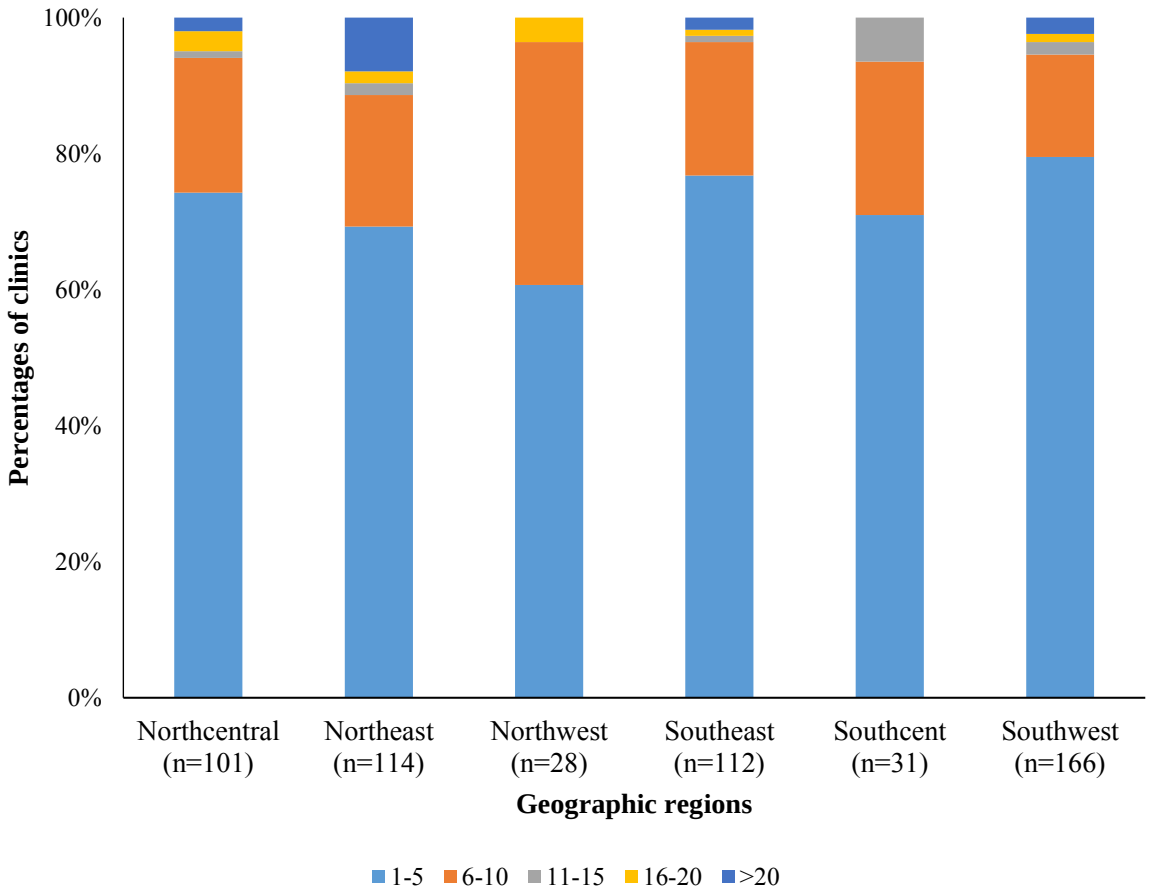

Figure 3.1. Percentages of number clinics (Y axis) were calculated from different size of clinics based on the number clinicians. The information was collected from six different geographic regions (X axis) among the US.

3.3.2 Antimicrobials prescription behaviors

Of the 823 cases, antimicrobials were prescribed in 765 cases (93.2%, 95% CI 89.7-94.8). The relationship between duration of infection and the timing of sample collection is shown in Figure 3.2. Most patients (47.6%) received their first antimicrobial dose after the sample was collected (Figure 3.2), and presented clinical signs within one week. Five hundred sixty patients (73.7%; 560/765) had received empirical antimicrobial treatment started before (63.2%; 483/765) or after (10.5%; 91/765) the results of the susceptibility were known. The day

0 or the day at antimicrobial prescription was the highest proportion among before or after collected samples ($P<0.05$).

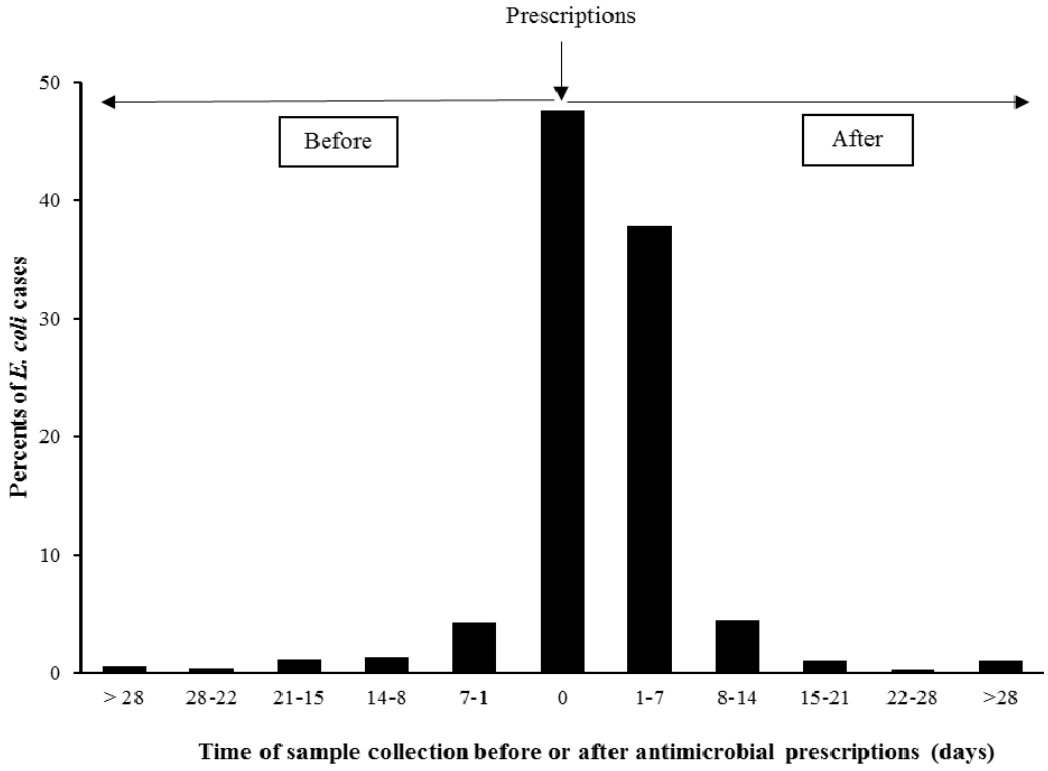

Figure 3.2 The relationship between duration of infection (N=823) and the timing of sample collection. The Y axis represents the percentages of cases for each time range group (X axis).

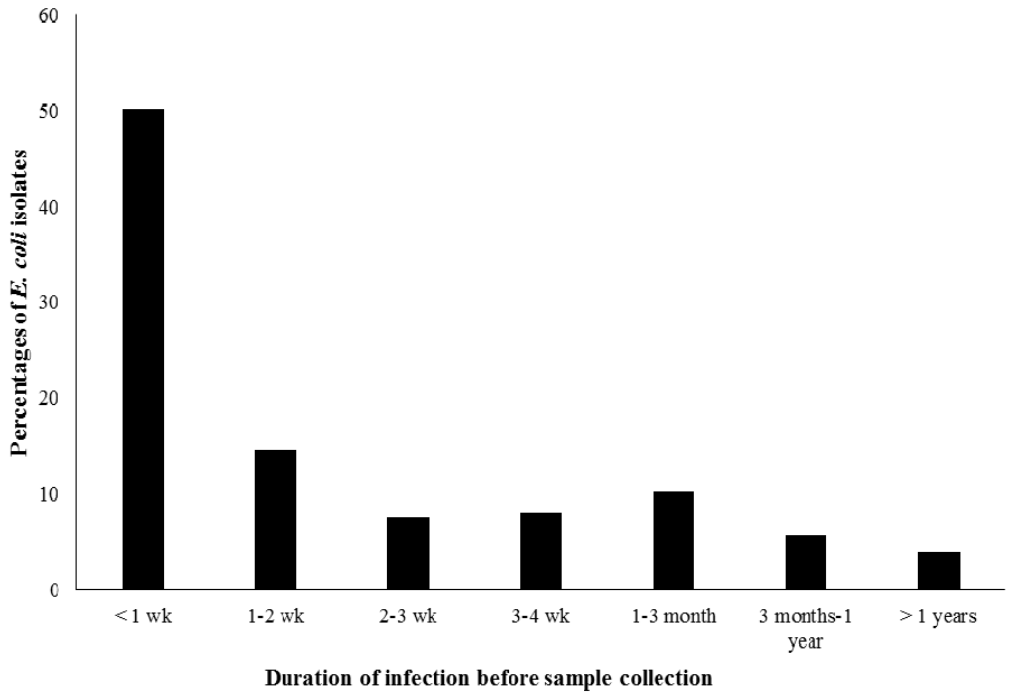

Figure 3.3 The distribution of *E. coli* isolates (total N=823) were collected based on the time when clinical signs presented. The Y axis represents the percentages of cases for each duration group (X axis).

Twenty-four antimicrobials (eight antimicrobial classes) had been used to treat *E. coli* infection. The most 10 common antimicrobial used and their dosing regimens are summarized in Table 3.1. The two drugs most commonly prescribed drugs were amoxicillin-clavulanic acid (190/760; 24.8%), and enrofloxacin (170/760; 22.4%) (Table 3.1). The least common prescribed drugs were nitofurantoin (0.1%), tylosin (0.1%), gentamicin (0.2%), amikacin (0.3%), and clindamycin (0.8%). Generally, the proportions of antimicrobial use-e.g. amoxicillin-clavulanic acid, 1st generation cephalosporins, ciprofloxacin, marbofloxacin and cefovecin- were significantly different (P<0.05) between dogs and cats (Table 3.1). Clinicians used a variety of dosing regimens for each of the antimicrobial uses. Most antimicrobial dosing regimens were

within the range of published guidelines for urinary tract infection [9]. The means of dose did not statistically differ between dogs and cats ($P>0.05$).

Since the majority of isolates were cultured from the urinary tract, the numbers for other infection sites were limited, precluding inferential statistical analysis. Therefore to facilitate analysis, infection locations were classified as to either urine or non-urine. When considered by infection location, amoxicillin-clavulanic acid was prescribed more commonly than any other antimicrobial (28.7%) for treatment of (presumed) uropathogens ($P<0.001$, Table3.1). The means of dose were not significantly difference between urine and non-urine.

Drugs	N (%)	Source	N (%)[a]	Dose Interval (hours)	N	Mean of dose ± S.D. (mg/kg)	Species	N (%)[b]	Dose Interval (hours)	N	Mean of dose ± S.D. (mg/kg)
Amoxicillin-clavulanic acid	190 (24.8%)	Urine	166 (28.7%)[e]	8	6	16.2±8.6	Dogs	127 (22.2 %)	8	2	21.6±1.7
				12	158	14.9±5.4			12	122	15.1±6.0
				24	1	20.4			24	3	18.5±5.7
		non-Urine	24 (12.8%)	8	1	22..8	Cats	63 (32.8%)[d]	0	0	N/A
				12	12	14.9±7.9			12	58	14.4±5.0
				24		17.3±7.4			24	5	9.4±6.0
Enrofloxacin	170 (22.2%)	Urine	128 (22.2%)	12	46	5.2±4.7	Dogs	132 (23.0%)	12	60	4.7±3.8
				24	80	5.6±2.8			24	71	6.0±2.4
		non-Urine	42 (22.3%)	12	21	3.9±1.6	Cats	38 (19.8%)	12	7	6.3±5.8
				24	21	6.9±2.9			24	30	5.7±3.7
1st generation cephalosporins[f]	68 (8.9%)	Urine	47 (8.1%)	8	6	20.6±5.9	Dogs	63 (11.0%)[c]	8	10	23.2±6.4
				12	35	22.4±8.6			12	47	22.8±8.0
				24	6	16.0±7.9			24	5	13.9±7.2
		non-Urine	21 (11.2%)	8	5	26.7±5.0	Cats	5 (2.6%)	8	1	25
				12	14	24.7±6.0			12	2	29.4±2.0
				24	1	22			24	2	23.3±2.4
Amoxicillin	66 (8.6%)	Urine	57 (10.0%)	8	2	12.7±1.9	Dogs	51 (8.9%)	8	2	12.7±1.9
				12	53	16.7±6.7			12	48	17.6±6.3
		non-Urine	9 (4.5%)	12	9	15.4±5.7	Cats	15 (7.8%)	12	15	12.8±6.5
Ciprofloxacin	60 (7.8%)	Urine	32 (5.5%)	8	0	N/A	Dog	59 (10.3%)[d]	8	1	2.4
				12	24	15.3±9.0			12	40	14.1±7.4
				24	8	14.5±10.0			24	15	17.7±11.2
		non-Urine	28 (14.9%)	8	1	2.4	Cats	1 (0.5%)	8	0	N/A

				12	17	11.9±3.8			12	1	5
				24	7	22.0±12.1				0	N/A
Cefpodoxime	45 (5.9%)	Urine	35 (6.1%)	24	35	7.2±2.8	Dogs	45 (7.9%)	24	45	7.7±3.2
		non-Urine	10 (5.3%)	24	10	9.4±3.8	Cats	0			
Marbofloxacin	42 (5.5%)	Urine	32 (5.5%)	12	3	4.4±1.3	Dogs	22 (3.8%)	12	1	5.6
				24	27	4.7±5.3			24	21	3.8±1.3
		non-Urine	10 (5.3%)	12	0	N/A	Cats	20 (10.4%)[d]	12	2	3.8±0.9
				24	10	4.5±1.5			24	16	5.7±6.7
Cefovecin	38 (5.0%)	Urine	33 (5.7%)	q14d	33	9.36±2.5	Dogs	5 (0.9%)	q14d	5	7.9±0.9
		non-Urine	5 (2.7%)	q14d	5	8.3±1.0	Cats	33 (17.2%)[e]	q14d	33	9.1±1.3
TMPS	19 (2.5%)	Urine	16 (2.8%)	12	12	24.6±9.1	Dogs	18 (3.1%)	12	12	22.7±9.4
				24	4	24.3±2.9			24	5	25.5±3.6
		non-Urine	3 (1.6%)	12	2	14.6±0.4	Cats	1 (0.5%)	12	1	28
				24	1	30.2					
Orbifloxacin	13 (1.7%)	Urine	11 (1.9%)	12	2	2.8±1.0	Dogs	7 (1.2%)	12	1	2.1
				24	9	6.5±6.4			24	6	3.7±1.7
		non-Urine	2 (1.1%)	24	2	4.7±3.0	Cats	6 (3.1%)	12	1	3.5
									24	5	9.2±7.9
Others[g]	54 (7.1%)	Urine	21 (3.4%)	N/A	N/A	N/A	Dogs	44 (7.7%)	N/A	N/A	N/A
		non-Urine	28 (15.3%)	N/A	N/A	N/A	Cats	10 (5.2%)	N/A	N/A	N/A

Table 3.1 Numbers of antimicrobial selections and dose regimens were documented by species and infection location.

a Percentages of antimicrobial uses were calculated from total number of urine (n=578) or non-urine (n=187) samples.

b Percentages of antimicrobial uses were calculated from total number of dogs (n=573) or cats (n=192) samples.

To identify factors associated with antimicrobial selection, logistic regression models were computed using clinic information (geographic region, and size of clinic), demographic data (species, gender, and age), history of infection (collection site, and severity), and other patient factors (Tables 3.2). Since some antimicrobials were prescribed too infrequently to analyze, analysis was limited to those antimicrobials which prescribed at least 5% of the time. Neither region nor size of clinic was associated with antimicrobial use. In general, female patients tended to be more exposed to antimicrobials than males, with odds ratio (OR) 2.4 (95% CI 1.1-5.2; $P<0.05$; Table 3.2). Species was generally a factor for antimicrobial selection; the exceptions being enrofloxacin and amoxicillin ($P<0.05$; Table 3.2). Patients with urinary tract infections were more likely to be prescribed amoxicillin-clavulanic acid than other infection sites (OR=5.7, 95% CI 2.5-13.1; $P<0.001$). Patients with life-threatening infections were prescribed 1st generation cephalosporins (cephalexin; 4/18) more than those in others severities (OR=8.1, 95% CI 1.7-3.9; $P<0.05$). Invasive procedures were a factor for amoxicillin-clavulanic acid (OR=4.0, 95% CI 1.3-12.6; $P<0.05$) and ciprofloxacin (OR=2.9, 95% CI 1.0-8.3; $P<0.05$). The patients that had received antimicrobial therapy prior to sample collection were significantly more likely to have been treated with marbofloxacin (OR=2.7, 95% CI 1.0-7.3; $P<0.05$).

Percentages were significantly higher than the other variable with [c] $P< 0.05$, [d] $P<0.01$, and [e] $P<0.0001$.

[f] 1st generation cephalosporins included cefadroxil, cephalexin, and cephalothin.

[g] Other antimicrobials were ampicillin, doxycycline, chloramphenicol, clindamycin, amikacin, ceftazidime, meropenen, difloxacin, gentamicin, imipenem, nitrofurantoin, tetracycline, ticarcillin-clavulanic acid, and tylosin.

Factors		Antimicrobials	AMC	ENR	CEP	AMX	CIP	CFP	MBF	CFV
Practices										
Regions	NC	N/A	2.0 (0.6-7.0)	0.5 (0.2-1.5)	N/A	N/A	0.2 (0.03-1.2)	0.3 (0.02-4.2)	0.8 (0.1-8.5)	0.1 (0.01-1.8)
	NE	N/A	1.3 (0.4-4.7)	0.3 (0.1-1.0)	N/A	N/A	0.4 (0.1-2.0)	1.7 (0.2-14.7)	0.6 (0.1-6.3)	0.7 (0.1-7.2)
	SC	N/A	1.9 (0.4-8.7)	0.4 (0.1-1.5)	N/A	N/A	0.3 (0.03-2.0)	4.3[a] (0.4-44.2)	2.5 (0.2-30.8)	0.4 (0.02-8.7)
	SE	N/A	1.5 (0.4-5.1)	0.3 (0.1-0.9)	N/A	N/A	0.8 (0.2-3.5)	1.6 (0.2-14.2)	0.6 (0.1-6.4)	0.3 (0.02-3.2)
	SW	N/A	1.4 (0.4-4.8)	0.5 (0.2-1.3)	N/A	N/A	0.8 (0.2-3.0)	1.5 (0.2-12.9)	0.7 (0.1-6.9)	0.2 (0.02-2.6)
	NW	1 (reference)								
Size of clinic	1-5	0.9 (0.1-8.3)	N/A	N/A	1.6 (0.2-13.6)	N/A	N/A	N/A	N/A	N/A
	6-10	0.8 (0.1-8.4)	N/A	N/A	1.5 (0.2-14.4)	N/A	N/A	N/A	N/A	N/A
	11-15	0.2 (0.01-3.9)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	16-20	0.5 (0.02-11.9)	N/A	N/A	3.4 (0.2-75.5)	N/A	N/A	N/A	N/A	N/A
	>20	1 (reference)								
Demographic data										
Species	Canine	3.3 (1.2-9.3)	1 (reference)	1.7 (0.9-3.4)	3.4[a] (1.3-8.9)	1.5 (0.6-3.5)	14.6[b] (2.0-107.8)	N/A	1 (reference)	1 (reference)
	Feline	1 (reference)	2.4[b] (1.4-4.2)	1 (reference)					3.4[b] (1.4-8.4)	23.4[c] (6.8-80.4)
Gender	Female	2.4[a] (1.1-5.2)	1.2 (0.7-2.1)	0.8 (0.5-1.5)	2.7[a] (1.2-6.1)	1.3 (0.6-3.0)	0.7 (0.4-1.6)	1.6 (0.6-4.1)	0.8 (0.3-2.4)	0.5 (0.2-1.6)
	Male	1 (reference)								
Age	0-1	N/A	5.3[‡] (1.9-15.1)	0.8 (0.2-2.7)	0.6 (0.1-4.2)	N/A	0.3 (0.02-5.7)	N/A	N/A	N/A
	1.1-3	0.4 (0.1-2.9)	2.2 (0.7-6.4)	0.7 (0.2-2.3)	1.9 (0.3-11.6)	N/A	1.5 (0.1-16.2)	N/A	N/A	N/A
	3.1-5	0.3 (0.04-2.3)	3.1 (1.0-9.1)	0.4 (0.1-1.5)	1.5 (0.2-9.5)	N/A	0.9 (0.1-10.6)	N/A	N/A	N/A
	5.1-7.5	0.4 (0.1-2.9)	0.9 (0.3-2.8)	1.1 (0.4-3.3)	1.0 (0.2-5.6)	N/A	1.7 (0.2-16.5)	N/A	N/A	N/A
	7.6-10	1.0 (0.1-7.4)	1.4 (0.6-3.7)	1.2 (0.4-3.3)	1.2 (0.2-6.6)	N/A	2.3 (0.3-20.7)	N/A	N/A	N/A

	10.1-12.5	0.6 (0.1-3.6)	1.3 (0.5-3.2)	1.0 (0.32.6)	0.8 (0.1-4.9)	N/A	0.8 (0.1-7.7)	N/A	N/A	N/A
	12.6-15	0.8 (0.1-5.4)	1.4 (0.6-1.2)	1.1 (0.4-2.8)	1.8 (0.3-9.1)	N/A	1.4 (0.1-13.2)	N/A	N/A	N/A
	>15	1 (reference)								
History of infection										
location	Urine	1.3 (0.4-4.1)	5.7[c] (2.5-13.1)	0.9 (0.5-1.5)	0.7 (0.3-1.5)	2.0 (0.7-5.7)	1.0 (0.4-2.3)	1.5 (0.5-4.1)	0.8 (0.2-2.9)	3.8 (0.4-35.5)
	Non-urine	1 (reference)								
Severity	Mild	1.5 (0.4-6.6)	1.7 (0.8-3.7)	0.9 (0.4-1.9)	1.6 (0.5-5.6)	0.4 (0.1-1.2)	N/A	N/A	1.9 (0.4-9.0)	0.9 (0.1-7.6)
	Moderate	2.3 (0.6-8.5)	1.4 (0.7-2.8)	0.8 (0.4-1.5)	2.0 (0.7-6.1)	0.5 (0.2-1.2)	N/A	N/A	0.8 (0.2-3.7)	3.6 (0.7-19.1)
	Severe	2.0 (0.4-9.8)	1.1 (0.4-2.6)	0.9 (0.4-2.2)	2.8 (0.8-9.5)	0.3 (0.1-1.2)	N/A	N/A	1.5 (0.3-8.3)	0.8 (0.1-7.2)
	Life-threatening	N/A	0.5 (0.1-3.6)	0.2 (0.02-1.9)	8.1* (1.7-39.9)	0.9 (0.1-6.2)	N/A	N/A	3.5 (0.2-51.0)	3.0 (0.2-56.2)
	No clinical sign	1 (reference)								
Hospitalized	Yes	0.4 (0.2-1.0)	0.7 (0.3-1.7)	1.9 (0.9-4.3)	1.1 (0.3-3.9)	1.1 (0.3-3.8)	1.3 (0.4-4.4)	1.0 (0.3-3.7)	0.2 (0.01-1.7)	0.4 (0.1-1.7)
	No	1 (reference)								
Invasive procedure	Yes	0.6 (0.1-3.9)	4.0[a] (1.3-12.6)	0.5 (0.1-2.0)	0.1 (0.01-1.1)	2.5 (0.5-13.1)	2.9[a] (1.0-8.3)	1.3 (0.2-8.3)	1.2 (0.1-13.3)	N/A
	No	1 (reference)								
Other illness	Yes	2.0 (0.8-4.8)	0.0 (0.6-1.5)	1.1 (0.7-1.8)	0.8 (0.4-1.6)	0.9 (0.5-1.8)	1.4 (0.7-2.9)	0.7 (0.3-1.5)	0.3[a] (0.1-1.0)	2.3 (0.8-7.0)
	No	1 (reference)								
Previous Antimicrobial	Yes	0.7 (0.2-2.5)	0.8 (0.4-1.6)	1.0 (0.5-1.8)	0.6 (0.2-1.5)	0.9 (0.3-2.3)	1.3 (0.5-3.1)	1.1 (0.4-3.1)	2.7[a] (1.0-7.3)	2.0 (0.5-7.7)
	No	1 (reference)								

Table 3.2 Odds ratios for the association of percent of antimicrobial prescription.

Factors were significantly associated as [a] *P< 0.05*, [b] *P<0.01*, and [c] *P<0.0001*.

To identify factors associated to prescribed antimicrobials, logistic regression models were computed using clinic information (geographic region, and size of clinic), demographic data (species, gender, and age), history of infection (collection site, and severity), and other patient factors (Tables 3.2). Since prescribed proportion of some antimicrobials were too low to analyze, this study selected antimicrobials which had more than 5% prescription proportion to further identify risk factors. Neither region nor size of clinic was associated with any antimicrobials prescription. For demographic data, age was not a factor for antimicrobial prescription. In general, female patients tended to be more exposed to antimicrobials than males with odd ratio (OR) 2.4 (95% CI 1.1-5.2; $P<0.05$). Species was a risk factor for most antimicrobial prescription; the exceptions being enrofloxacin and amoxicillin ($P<0.05$). As such, canine patients were more likely to be prescribed than from feline patients for 1st generation cephalosporins (OR=3.4, 95% CI 1.3-8.9; $P<0.05$) and ciprofloxacin (OR=14.6, 95% CI 2.0-107.8; $P<0.01$). In contrast, amoxicillin-clavulanic acid (OR=2.4, 95% CI 1.4-4.2; $P<0.01$), marbofloxacin (OR=3.4, 95% CI 1.4-8.4; $P<0.01$) and cefovecin (OR=23.4, 95% CI 6.8-80.4; $P<0.001$) were more prescribed in cats than dogs. Patients with urinary tract infections were more likely to be prescribed amoxicillin-clavulanic acid than other infection cites (OR=5.7, 95% CI 2.5-13.1; $P<0.001$). Patients in life-threatening infections were prescribed 1st generation cephalosporins more than those in others severities (OR=8.1, 95% CI 1.7-3.9; $P<0.05$). Invasive procedures were a factor for amoxicillin-clavulanic acid (OR=4.0, 95% CI 1.3-12.6; $P<0.05$) and ciprofloxacin (OR=2.9, 95% CI 1.0-8.3; $P<0.05$). Previous antimicrobial therapy within three months were a risk factor for marbofloxacin (OR=2.7, 95% CI 1.0-7.3; $P<0.05$).

3.3.3 Risks of resistance to an antimicrobial after exposure to that same antimicrobial

For the majority of cases, the antimicrobial selected was found to be susceptible on culture (Table 3). Based on 12 antimicrobial categories, the proportion of MDR (49%) and SDR (49%) isolates was greater than XDR (15%; $P<0.0001$). Most cases of XDR infection was treated with enroflaxacin (13/14; OR=2.2, $P=0.03$; table 3.3).

Antimicrobial selection	Resistance of *E. coli* isolates		OR (95% CI)	*P* value	Type of resistance			OR (95% CI)	*P* value
	Yes	No			SDR	MDR	XDR		
Amoxicillin-clavulanic acid									
Yes	45 (23.7%)	145 (76.3%)			120 (63.2%)	170 (89.5%)	0		
No	228 (39.6%)	348 (60.4%)	0.5 (0.3-0.7)	<0.01	263 (45.7%)	298 (51.8%)	14 (2.4%)	0.5 (0.4-0.7)	<0.01
Enrofloxacin									
Yes	10 (5.9%)	160 (94.1%)			79 (43.4%)	90 (49.5%)	13 (7.1%)		
No	58 (9.7%)	538 (90.3%)	0.6 (0.3-1.2)	0.2	304 (52.2%)	277 (47.6%)	1 (0.2%)	2.2 (1.1-3.5)	0.03
1st generation cepharosporin									
Yes	67 (98.5%)	1 (1.5%)			33 (48.5%)	21 (30.9%)	14 (20.6%		
No	676 (96.8%)	22 (3.2%)	N/A		343 (50.3%)	339 (49.7%)	0	N/A	
Amoxicillin									
Yes	14 (21.2%)	52 (78.8%)			49 (74.2%)	17 (25.8%)	0		
No	317 (45.3%)	383 (54.7%)	0.3 (0.2-0.6)	<0.01	334 (47.9%)	350 (50.1%)	14 (2.0%)	0.3 (0.2-0.6)	0.0003

Table 3.3 Univariate analysis of therapies, including amoxicillin-clavulanic acid, enrofloxacin, 1st generation cephalosporins* (cefadroxil, cephalexin, and cephalotin), and amoxicillin, as risk factors for antimicrobial-specific resistance and type of resistance (SDR, MDR, and XDR) in 766 *E. coli* strains.

3.3.4 Success rate of antimicrobial therapy

To access the efficacy of antimicrobial therapy, success rates of antimicrobial therapy were depicted in Figure 3.4. Resolved infection were interpreted as successful therapy whereas antimicrobial therapy was considered inefficacies if infections were persistent or recurrent. The rates of antimicrobial response among antimicrobials use were not significantly difference (P>0.05). The highest success rate was cefovecin (25/31; 81%), followed by amoxicillin (41/53; 77%), and the least was 1st generation cephalosporin (32/53; 60%) (P<0.05; Figure 3.4).

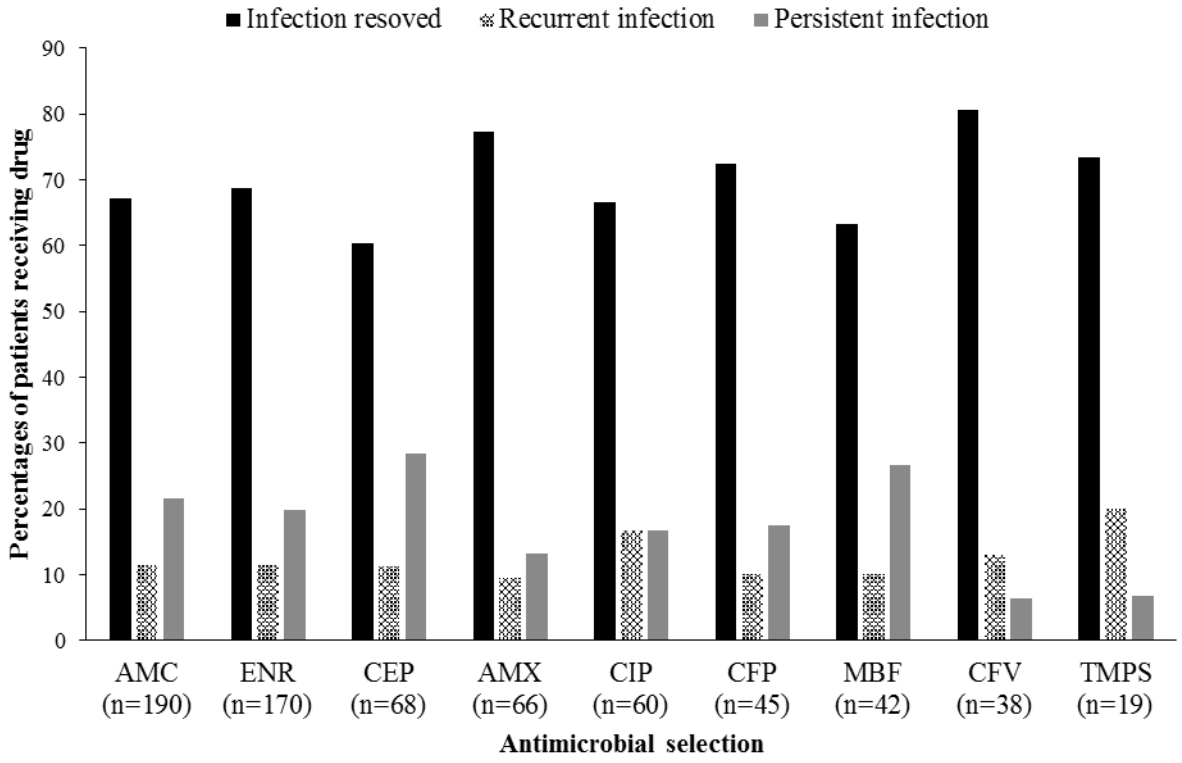

Figure 3.4 Percentages of *E. coli* infection cases responded to antimicrobial therapy. Success rates (percentages of infection resolved) of antimicrobial therapy was not significantly different (P>0.05).

Abbreviations: amoxicillin-clavulanic acid (AMC), 1st generation cephalosporins (CEP), amoxicillin (AMX), enrofloxacin (ENR), ciprofloxacin (CIP), cefpodoxaime (CFP), marbofloxaicn (MBF), cefovecin (CFV), and sulfamethoxazole/trimethoprim (TMPS).

3.4 Discussion

This is the first retrospective study that demonstrated the patterns of antimicrobial use and prescribing behavior of veterinarians providing care to dogs or cat. The availability of similar studies is limited because proper information enabling a detailed description of the antimicrobial use in practice conditions is often missing. Although a few studies have investigated the prescription behaviors of small animal practitioners, most of them were conducted at veterinary teaching hospitals. Our study is one of the few sources in the U.S. that provides a relevant insight into the patterns of antimicrobial usage in dogs and cats to treat *E. coli* infections.

Our findings showed that antimicrobial selection was most commonly empirical, being implemented prior to receipt of the susceptibility data (74%). It is a cause of concern that bacteriological culture and susceptibility testing are rarely used before antimicrobial selection. Our findings showed that the most common antimicrobial use for UTI treatment was amoxicillin-clavulanic acid (29%), and enrofloxacin (22%). According to antimicrobial use guideline for treatment of UTI, recommended antimicrobials for initial therapy of uncomplicated UTI were amoxicillin (11-15 mg/kg PO q 8h), or trimethoprim-sulfonamide (15 mg/kg PO q12h); moreover, amoxicillin-clavulanic is not recommended [9]. Since *E. coli* was intrinsically resistant to clindamycin and tylosin [18], this study found that their use for treatment of *E. coli*

infection was inappropriate. Thus, this study demonstrated that the empirical choice of an antimicrobial agent complies fairly well with the prudent guidelines.

Widespread usage of amoxicillin-clavulanic acid was observed in both dogs and cats. These findings were consistent with previous studies in Australia, Italy, Finland, and UK [19] [20] [21] [22] [23]. Since amoxicillin-clavulanic acid is a broad-spectrum, inexpensive drug with few side effects, its use as a first choice without culture and susceptibility testing is considered overused by some authors [24]. However, the addition of a β-lactamase inhibitor (clavulanic acid) generally does not increase efficacy of aminopenicillins against *E. coli* infection and this was demonstrated by our study [9]. This study suggested that the addition of clavulanic acid is considered unnecessary for treatment against *E. coli* urinary tract infections. Amoxicillin was the fourth most prescribed antimicrobial and was prescribed in a high proportion of urinary tract infections. The most common indications for amoxicillin use are for an unknown target infection and first choice for uncomplicated cystitis [9]. Amoxicillin is a relatively safe, inexpensive, board-spectrum antimicrobials, which makes it a relatively good choice for empirical use.

Enrofloxacin was the second most commonly prescribed antimicrobial in both dogs and cats; also both UTI (22%) and non-UTI (22%). Enrofloxacin was the first of the veterinary fluororquinolone to be approved for use in dogs and cats so this might provide clinicians ease to use. Other fluororquinolones are currently approved for oral use in small animals in the US: orbifloxacin (dogs and cats), difloxacin (dogs), and marbofloxacin (dogs and cats) [25], this fluororquinolones are less commonly used when compare to enrofloxacin because these fluororquinolones has been approved shorter period in the U.S. However, fluoroquinolones are recommended as a second line treatment for otitis media, severe pneumonia, peritonitis and pyelonephritis [26] [9]. Moreover, this study reported that dose of enrofloxacin for canine UTI

was 5.6±2.8 mg/kg PO q24h, which differ from the recommended dose (10-20 mg/kg PO q24h) for guideline of UTI [9]. Minimizing the exposure and spread of fluororquinolone resistance is important since resistance has developed into multidrug resistance [27] [28]. The use of fluororquinolone without culture and susceptibility testing as a first choice and incorrect dose were inappropriate.

Cefovecin, a third-generation cephalosporins, recently licensed for veterinary use was the third most commonly used systemic antimicrobial in feline patients. This study is similar to previous study in the UK [23]. This is probably due to the convenience of the antimicrobial administration (i.e. long-acting injection with a 14-day duration), which can be used by veterinary practitioners when compliance by owners cannot otherwise be guaranteed. Cefovecin was not widely used in dogs, possibly due, in part, to the high cost involved when treating medium to large animals and the ease to administer oral treatments this species when compared to cats. Cats were more likely to be exposed to critically important antimicrobials-i.e. fluororquinolone, and 4rd generation cephalosporins (cefovecin) [29]. This may be due to the antimicrobial preparation currently licensed for use in cats in the US.

To assess appropriate usage of antimicrobials in small animal practice, complete and accurate clinical data would be required. Other than data concerning the animal patients, antimicrobial drugs, dose, frequency, and duration of treatment prescribed were includes in this study. This study suggested that the efficacy of antimicrobial therapy should be determined by integration of pharmacokinetic and pharmacodynamics. The pharmacokinetic/ pharmacodynamics parameter is the ability of an antimicrobial dosing regimens to inhibit pathogenic bacteria [30]. However, the pharmacokinetic/ pharmacodynamics characteristics is beyond the scope of this paper. Nevertheless, this study provided the patterns of antimicrobial

use, and supported the need of evaluation of the appropriateness of antimicrobial use by pharmacokinetic/ pharmacodynamics parameter.

Our data are subject to several limitations. This study was conducted in dogs and cats upon a single visit at private veterinary practice in the US; our data relied on questionnaires to determine an infection history, and patients' conditions in which veterinarians may have different levels of diagnoses such as severity of infections. Despite these limitations our findings strongly suggest that veterinary medical clinicians frequently prescribe antimicrobials without corresponding documentation of culture and susceptibility testing. This study also demonstrates the challenges in evaluating the appropriateness of antimicrobial use in specific clinical cases. Therefore, information of antimicrobial therapy is vital and frequent follow-up is needed in order to be able to do comparisons between treatment practices and antimicrobial resistance.

4.5 Conclusion

Generally, clinicians use empirical antimicrobial therapy and a variety of dosing regimens of antimicrobials. Our findings reported that the selection of antimicrobial is inappropriate for empirical antimicrobial for treatment of urinary tract infection with *E. coli*, and lead to high rate (>20%) of therapeutic failure. Thus, our study suggests that improving the procedures of antimicrobial prescription in companion animals may possibly lead to a decrease in the therapeutic failure and prevalence of emerging antimicrobial resistance.

4.6 References

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CHAPTER 4

PHYLOGENETIC ORIGIN AND VIRULENCE GENOTYPE IN RELATION TO ANTIMICROBIAL RESISTANCE AMONG *ESCHERICHIA COLI* ISOLATES FROM CANINE URINARY TRACT INFECTIONS

4.1 Introduction

UTIs due to *E. coli* cause considerable morbidity, mortality (pyelonephritis), and increased health care cost in both human and veterinary medicine. ExPEC includes as a subset UPEC isolates that acquire VFs necessary for survival in the urinary tract [1]. ExPEC and commensal *E. coli* typically differ with respect to phylogenetic background and virulence traits. Pathogenic *E. coli* strains derive from phylogenetic group B2 and D (a lesser extent from group D) [2]. In contrast, commensal *E. coli* are characteristically from phylogenetic group A. Moreover, pathogenicity has been correlated with the presence of genes encoding VFs [3].

Virulence determines whether or not an isolate will cause pathogenesis to the hosts. UPEC pathogenesis begins with bacterial adhesins (*pap, fim*) that facilitate penetration of uroepithelial cells. Toxins (e.g, cytotoxic necrotizing factor [*cnf*], hemolysins [*hyl*]) cells, and iron scavenging is mediated via siderophores *(ireA*, *iroN*), facilitating penetration. Important adhesins include Type 1 fimbreae (*fimAFGH*), FimH, necessary for recognition of mannose receptors of the uroepithelial cells and papillae (*pap*), which include genes necessary for renal colonization [4]. However, other adhesins signal structural changes in host cells that facilitate transition of the infection from an acute response to long-term persistence in the bladder wall (*papG, sfaS, foc*). The clinical signs associated with UTIs presumably vary with

the types of virulence gene expressed by the colonizing microbe-range from no clinical sign, mild, moderate, severe, to life-threatening (pyelonephritis and/or urosepsis). Pollakiuria, stranguria, dysuria, and inappropriate urination may be may be observed with lower UTI which were frequently diagnosed as mild to moderate infection [5].

The prevalence of antimicrobial resistance among canine and feline clinical isolates of *E. coli* has increased dramatically in recent years [6] [7]. Emerging resistance limits the utility of older agents, such as ampicillin, which results in increased reliance on newer agents, such as fluoroquinolones and extended-spectrum cephalosporins [8] [9]. Unfortunately, emerging resistance now threatens these newer agents as well [10]. Previously, among human clinical *E. coli* isolates, resistance to traditional antibiotics has been associated with decreased virulence (as assessed by VF profiles), phylogenetic groups other than virulence-associated group B2, and host immunocompromise [7]. Likewise, previous studies have suggested that antimicrobial-resistant *E. coli* tend to be less virulent than susceptible isolates, according to clinical signs and/ or virulent profiles [11]; however, these associations have rarely been studied in companion animals [12]. Additionally, that research focused on ExPEC resistances to a limited number of antimicrobials, mainly fluoroquinolones. Therefore, resistances to other antimicrobials that might be associated with phylogenetic origins and VFs are unknown.

An extensively antimicrobial-resistant *E. coli* sequence type ST131, has been recognized as an important human pathogen within the last several years [13]. *E. coli* ST131 also commonly occurs as a fluoroquinolone-resistant (FQ-R) but cephalosporin-susceptible pathogen [14]. ST131 has contributed importantly to the global emergence of the CTX-M-15 extended-spectrum beta-lactamase [15].

The purpose of the study reported was to assess the associations among antimicrobial resistance, molecular epidemiological virulence, and phylogenetic background within a collection of canine UPEC isolates from across the United States. We also investigated the incidence of *E. coli* ST 131.

4.2 Material and methods

4.2.1 E. coli Isolates.

In our library of 3172 *E. coli* isolates (chapter 2), 823 samples were accompanied had responded the questionnaires which have information of history infection (from Chapter 3). We stratified a sample pool of 405 canine *E. coli* isolates with urinary tract infection, and randomly selected 191 *E. coli* isolate to identify phylogenetic groups. For further molecular analyses, we randomly selected a total 68 from 191 isolates. Each isolate was classified two ways. Firstly, its association with clinical signs was classified in terms of severity ranging from absent (n=15), to mild (level 1; n=18), moderate (level 2; n=15), severe (level 3; n=17) or life-threatening (level 4; n=3). Secondly, each isolate was also designated as ABU (severity absent) or non-ABU (mild, moderate, severe, and life-threatening).

4.2.2 Antimicrobial susceptibility testing

All isolates were subjected to antimicrobial susceptibility testing from previous study (see chapter 2). Susceptibility testing was performed using 17 antimicrobials, representing 6 drug classes. Each isolate was designated as resistant "R" (MIC > the susceptible breakpoint), susceptible "S" (MIC ≤ the susceptible breakpoint) to each drug using CLSI guidelines. All isolates had been identified resistance phenotype (SDR, MDR, and XDR) from previous study (see chapter 2).

4.2.3 DNA extraction

Each isolate was grown up on trypticase soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ), and then incubated at 37ºC for 18 h. One *E. coli* colony was randomly selected to extract DNA. Bacterial DNA for PCR reaction were prepared by boiling a bacterial culture in 200 µl of PrepMan® preparation reagent (Applied Biosystem, Foster City, CA) at 95ºC for 10 min, and then centrifuges for 5 min at 4 ºC and 15,000 x g. The DNA solution was taken for 100 µl and kept -80 ºC freezer until the PCR performed.

4.2.4 Phylogenetic grouping

E. coli isolates were examined for their phylogenetic groups A, B1, B2, and D based on triplex PCR and the presence or absence of *chuA, yjaA* and TspE4.C2 genes as previously described [16].

4.2.5 Virulence gene detection

Oligonucleotide primers were used for the detection of 16 virulence genes which are used to identify extraintestinal *E. coli* (Table 4.1). Bacterial DNA amplification was performed by using the OmniMix™ HS lyophilized beads (Cepheid, Sunnyvale, CA) following the manufacturer's recommendations. Briefly, OmniMix master mix (containing per 25 µL reaction: 3U hot start *Taq* polymerase, 200 µM deoxynucleoside triphosphates, 4 mM $MgCl_2$ and 25 mM HEPES, pH 8.0) was mixed with the primers and added 1 µL of DNA template into each reaction, and the tubes were centrifuged for 30 s and then immediately placed into the preheated cycler. Amplification was performed on the Roche® LightCycler® Real-Time PCR System (Roche Applied Science, Indianapolis, IN). The cycler denatured the DNA for 5 min at 94°C and then amplified it by 35 cycles as follows:

denaturing for 30 s at 94°C, annealing of primers for 30 s at 60°C, and extension for 30 s at 72°C. Final extension occurred for 10 min at 72°C, and the reaction was concluded at 10°C. The PCR amplicons were visualized following electrophoresis on 1.25% agarose gel and staining with ethidium bromide. Amplicons were compared to negative control and positive reference strains.

4.2.6 E. coli ST 131 strains detection

All phylogenetic group B2 isolates were screened by ST131-specific single-nucleotide polymorphisms (SNPs) in *mdh* (i.e., C288T and C525T) and *gyrB* (i.e., C621T, C729T, and T735C) as previously described [17]. The primers used for the detection of *mdh* SNPs were mdh36_forward (5′-GTTTAACGTTAACGCCGGT-3′) and mdh36_reverse (5′-GGTAACACCAGAGTGACCA-3′), and the primers used for the detection of *gyrB* SNPs were *gyrB*47_forward (5′-CGCGATAAGCGCGAC-3′) and *gyrB*47_reverse (5′-ACCGTCTTTTTCGGTGGAA-3′). Amplification was done with the OmniMix™ HS lyophilized beads (Cepheid, Sunnyvale, CA) and a cycling protocol of 95°C for 10 min; then 32 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 2 min; and then a hold at 4°C.

Primers	Product size (bp)	Nucleotide sequences (5'-3')	Genbank accession no.	Reference
***papA*-F**	717	ATGGCAGTGGTGTCTTTTGGTG	X61239	[18]
***papA*-R**		CGTCCCACCATACGTGCTCTTC		
***papC*-F**	205	GTGGCAGTATGAGTAATGACCGTTA	X61239	[19]
***papC*-R**		ATATCCTTTCTGCAGGGATGCAATA		
***papG* allele I-F**	479	TCGTGCTCAGGTCCGGAATTT	X61239	[20]
***papG* allele I-R**		TCCAGAAATAGCTCATGTAACCCG		
***papG* allele I'-F**	461	CTACTATAGTTCATGCTCAGGTC	X61239	[18]
***papG* allele I'-R**		CTGACATCCTCCAACATTATCGA		
***papG* allele II-F**	190	GGGATGAGCGGGCCTTTGAT	M20181	[21]
***papG* allele II-R**		CGGGCCCCCAAGTAACTCG		
***papG* allele III-**	258	GGCCTGCAATGGATTTACCTGG	X61238	[18]
***papG* allele III-**		CCACCAAATGACCATGCCAGAC		
***focA*-F**	458	ATGCGTCYGCTGTCACCACGG	DQ301498	PS
***focA*-R**		GGCGTCGGCGTTGGCAATAC		
***focG*-F**	364	CAGCACAGGCAGTGGATACGA	DQ301498	[18]
***focG*-R**		GAATGTCGCCTGCCCATTGCT		
***sfaS*-F**	244	GTGGATACGACGATTACTGTG	S53210	[18]
***sfaS*-R**		CCGCCAGCATTCCCTGTATTC		
***iha*-F**	829	CTGGCGGAGGCTCTGAGATCA	AF126104	PS
***iha*-R**		TCCTTAAGCTCCCGCGGCTGA		
***fimH*-F**	508	TGCAGAACGGATAAGCCGTGG	AJ225176	[18]
***fimH*-R**		GCAGTCACCTGCCCTCCGGTA		
***cdtI*-F**	430	GAAAATAAATGGAACACACATGTCCG	AB472831	PS
***cdtI*-R**		AAATCTCCTGCAATCATCCAGTTA		

***sat*-F**	937	GCAGCTACCGCAATAGGAGGT	AF289092	PS
***sat*-R**		CATTCAGAGTACCGGGGCCTA		
***cnfI*-F**	974	ATCTTATACTGGATGGGATCATCTTGG	U42629	PS
***cnfI*-R**		GCAGAACGACGTTCTTCATAAGTAT		
***hlyD*-F**	904	CTCCGGTACGTGAAAAGGAC	AM690759	[18]
***hlyD*-R**		GCCCTGATTACTGAAGCCTG		
***ireA*-F**	254	GATGACTCAGCCACGGGTAA	AF320691	PS
***ireA*-R**		CCAGGACTCACCTCACGAAT		
***iroN*-F**	667	AAGTCAAAGCAGGGGTTGCCCG	CP001671	PS
***iroN*-R**		GACGCCGACATTAAGACGCAG		
***iut*-F**	302	GGCTGGACATCATGGGAACTGG	X05874	[21]
***iut*-R**		CGTCGGGAACGGGTAGAATCG		

Table 4.1 Primers for multiplex virulence genes polymerase chain reaction assay.

Abbreviation: F, forward; R, Reverse; PS, present study.

4.2.7 Statistical analysis

We used SAS software 9.2 (SAS Institute Inc., Cary, NC) to generate and calculate the data. All outcome variables are categorical including resistance phenotypes (SDR, MDR, or XDR), phylogenetic groups (A, B1, B2, and D), and virulence factor (present or absent genes). Proportional comparisons were made with regard to phylogenetic groups and prevalence of virulence genes, stratified by severity of clinical signs associated with the infections. Proportions were determined for each level of severity (1-5), and then again for no clinical signs (ABU) vs clinical signs (non-ABU). Isolates were regarded as independent samples, since each isolate was derived from a unique host and represented a separate

infection episode. Comparisons of proportions were tested using Pearson's χ^2 test or Fisher's exact (if n<5). All tests were two-tailed tests and $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Distribution of severity of clinical signs and phylogenetic groups

The percentages of 191 *E. coli* isolates from different severity of clinical signs are depicted Figure 4.1. The percentages of severity in order of percentage, were moderate (35%; 67/191), followed by mild (28%), severe (23%) and the least was life-threatening (1%). The ABU isolates represented 13% (24/191) of total canine UPEC strains.

Of the 191 canine UPEC studied, the majority of phylogenetic groups were D (40%; 76/191) and followed by B2 (31%), B1 (17%) and the least was group A (13%; Table 4.2 and Figure 4.1). Phylogenetic analysis in relation to severity of clinical signs revealed that the majority of severe isolates belonged to phylogroups B2 (50%; 22/44), followed by D (41%; 18/44). In contrast, isolates expressing no clinical signs mostly belonged to phylogroup D (63%; 15/24).

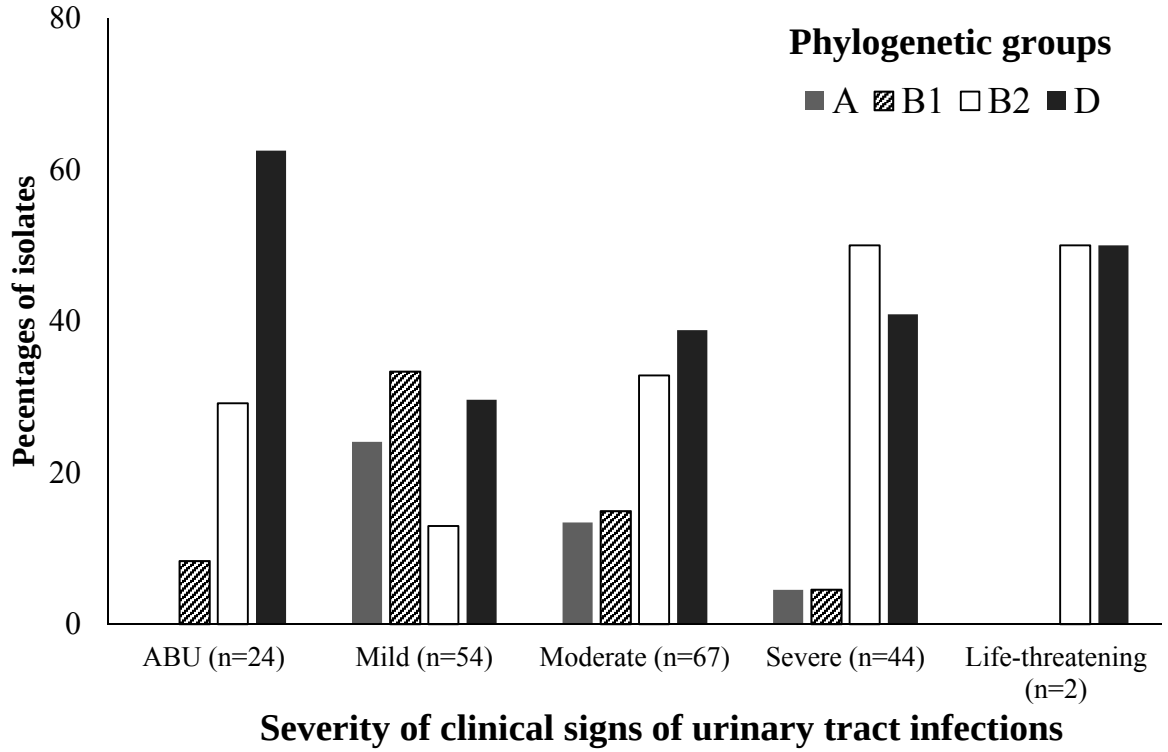

Figure 4.1 The percentages of severity of clinical signs among 191 *E. coli* isolates were identified on the basis of phylogenetic groups A, B1, B2, and D. The y axis represents the percentages of *E. coli* isolates for each severity of disease.

4.3.2 Relationship between phylogenetic group and phenotypic resistance

Among the 16 antimicrobials tested, resistance was most prevalent to CEP and DOX (100%; 191/191), followed by AMP (34%; 65/191), and the least prevalence was MEM (7/191; 4%). With regard to multidrug resistance profiles, 93 of the 191 (49%) isolates expressed SDR or resistant to 1-2 antimicrobial categories, 95 (50%) were MDR or resistant to 3-10 antimicrobial categories, and 3 (2%) were XDR or resistant to 11-12 antimicrobial categories (Table 4.2).

Resistant types	No. isolates (% of total)	Phenotype	No. isolates (% each resistant type)
SDR	93 (48.7%)	CD	93 (100%)
MDR	94 (49.2%)	ACD	11 (2.1%)
		CDH	11 (2.1%)
		XACD	5 (5.3%)
		XACDR	4 (4.2%)
		XCD	3 (3.2%)
		OCHD	3 (3.2%)
		ACDR	2 (2.1%)
		ACHD	2 (2.1%)
		CDG	2 (2.1%)
		OCD	2 (2.1%)
		PCD	2 (2.1%)
		XAOPZCDR	2 (2.1%)
		XAPCD	2 (2.1%)
		XACDRS	2 (2.1%)
		XATOPZCDR	2 (2.1%)
		[a]etc.	40 (42.1%)
XDR	3 (1.6%)	XAFTOPZCHDEGR	1 (33.3%)
		XAFOPZCHDEGRS	2 (66.7%)

Table 4.2 Multiple antimicrobial resistance pattern of 191 *E. coli* isolates.

[a]other phenotypes express one phenotype per one isolates. Abbreviation: A, ampicillin; C, cephalothin; D, doxycycline; E, Enrofloxacin; F, ciprofloxacin; G, gentamicin; H, chloramphenicol; M, meropenem; O, cefoxitin; P, cefpodoxime; R, ticarcillin-clavulanic acid; S, trimethoprim-sulphamethoxazole; T, cefotaxime; X, amoxicillin-clavulanic acid.

Phylotyping analysis in relation to resistance phenotype revealed that MDR isolates mostly belonged to phylogroups A (75%) and B1 (71%; Figure 4.2). In contrast, the SDR isolates were predominantly phylogroup B2 (64%). The proportion of MDR isolates in group B2 were significantly (P<0.05) less than that in group A and B1 (Figure 4.2).

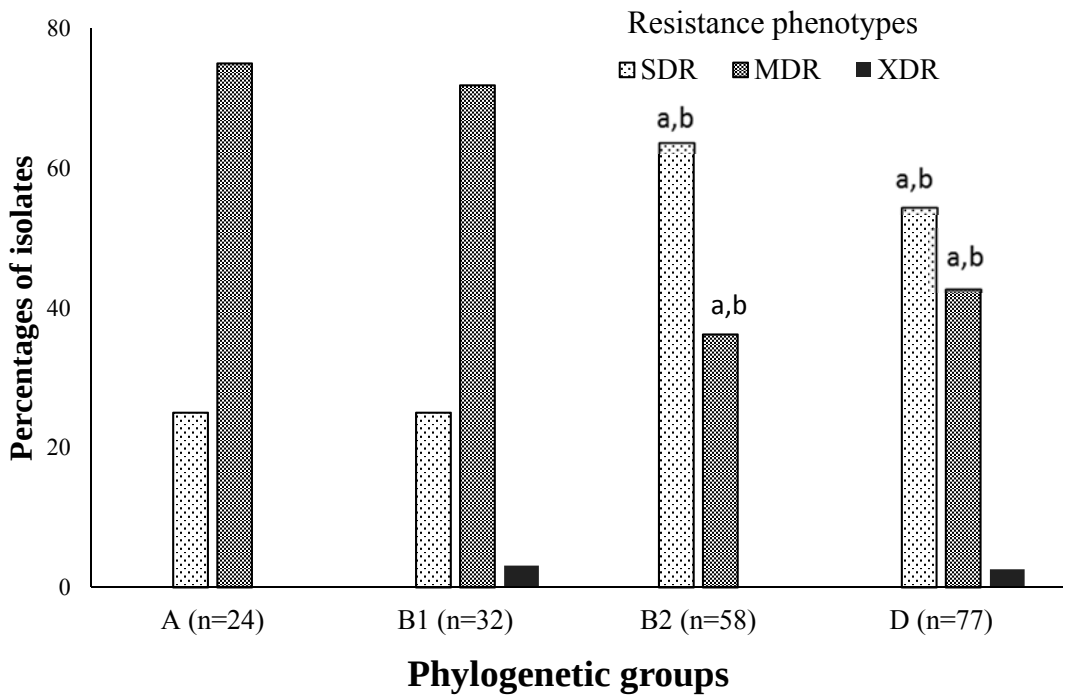

Figure 4.2 Antimicrobial resistance phenotypes (SDR: single drug resistance; MDR: multi drug resistance; XDR: extreme drug resistance) for 191 *E. coli* strains classified on the basis of phylogenetic groups (A, B1, B2, and D).

[a] The proportions were significantly different when compare to those in phylogenetic group A ($P<0.05$). [b] The proportions were significantly different when compare to those in phylogenetic group B1 ($P<0.05$).

4.3.3 Distribution of VF genes

From 191 *E. coli* isolates, we randomly selected 68 canine UPEC 16 (22%) ABU and 53 (78%) non-ABU, for identification of virulence genes. Multiplex PCR assays revealed that all of the 68 *E. coli* isolates exhibited at least one of the virulence genetic markers tested. The F1 fimbriae coding gene (*fimH*) was found in all isolates (100%; Table 4.3, Figure 4.3), followed by *focA* 86.8% (59/68), *iroN* 83.8% (57/68) and *hlyD* 72.1% (49/68), respectively.

Severity	ABU, No. (%)	Non-ABU, No. (%)				
		non-ABU	Mild	Moderate	Severe	Life-threatening
***E. coli* isolates (N=)**	16	52	18	15	17	2
Adhesin						
papA	7 (43.8)	27 (51.9)	12 (66.7)	6 (40.0)	8 (47.1)	1 (50.0)
papC	11 (68.8)	33 (63.5)	14 (77.8)	9 (60.0)	9 (52.9)	1 (50.0)
papG	6 (37.5)	28 (53.8)[a]	13 (72.2)	6 (40.0)	8 (47.1)	1 (50.0)
papG allele1	1 (6.2)	0	0	0	0	0
papG allele1'	0	1 (1.9)	0	0	0	1 (50.0)
papG allele2	0	1 (1.9)	0	1 (66.7)	0	0
papG allele3	11 (68.8)	36 (69.2)	15 (83.3)	11 (73.3)	10 (58.8)	0
fimH	16 (100)	52 (100)	18 (100)	15 (100)	17 (100)	2 (100)
focA	13 (81.3)	46 (88.5)	16 (88.9)	13 (86.7)	15 (88.2)	2 (100)
focG	12 (75.0)	28 (53.8)[a]	9 (50.0)	10 (66.7)	7 (41.2)	2 (100)
iha	3 (18.8)	3 (5.8)	1 (5.6)	2 (13.3)	0	0
sfaS	2 (12.5)	8 (15.4)	2 (11.1)	3 (20.0)	3 (17.6)	0
Toxins						
sat	2 (12.5)	3 (5.8)	1 (5.6)	2 (13.3)	0	0
cdtD	1 (6.3)	2 (3.8)	0	0	1 (5.9)	1 (50.0)
hylD	11 (68.8)	38 (73.1)	15 (83.3)	10 (66.7)	11 (64.7)	2 (100)
cnf1	7 (43.8)	36 (69.2)[a]	14 (77.8)	9 (60.0)	11 (64.7)	2 (100)
Siderophores						
iut	9 (56.3)	27 (51.9)	10 (55.6)	11 (73.3)	6 (35.3)	0
iroN	12 (75.0)	45 (86.5)	17 (94.4)	12 (80.0)	14 (82.4)	2 (100)
ireA	4 (25.0)	22 (42.3)[a]	4 (22.2)	9 (60.0)	7 (41.2)	2 (100)

Table 4.3 Distribution of virulence gene positive isolates categorized by five levels of severity.

[a] Within a row, value for the isolates from non-ABU was significantly greater ($P<0.05$) than the value for isolates from ABU.

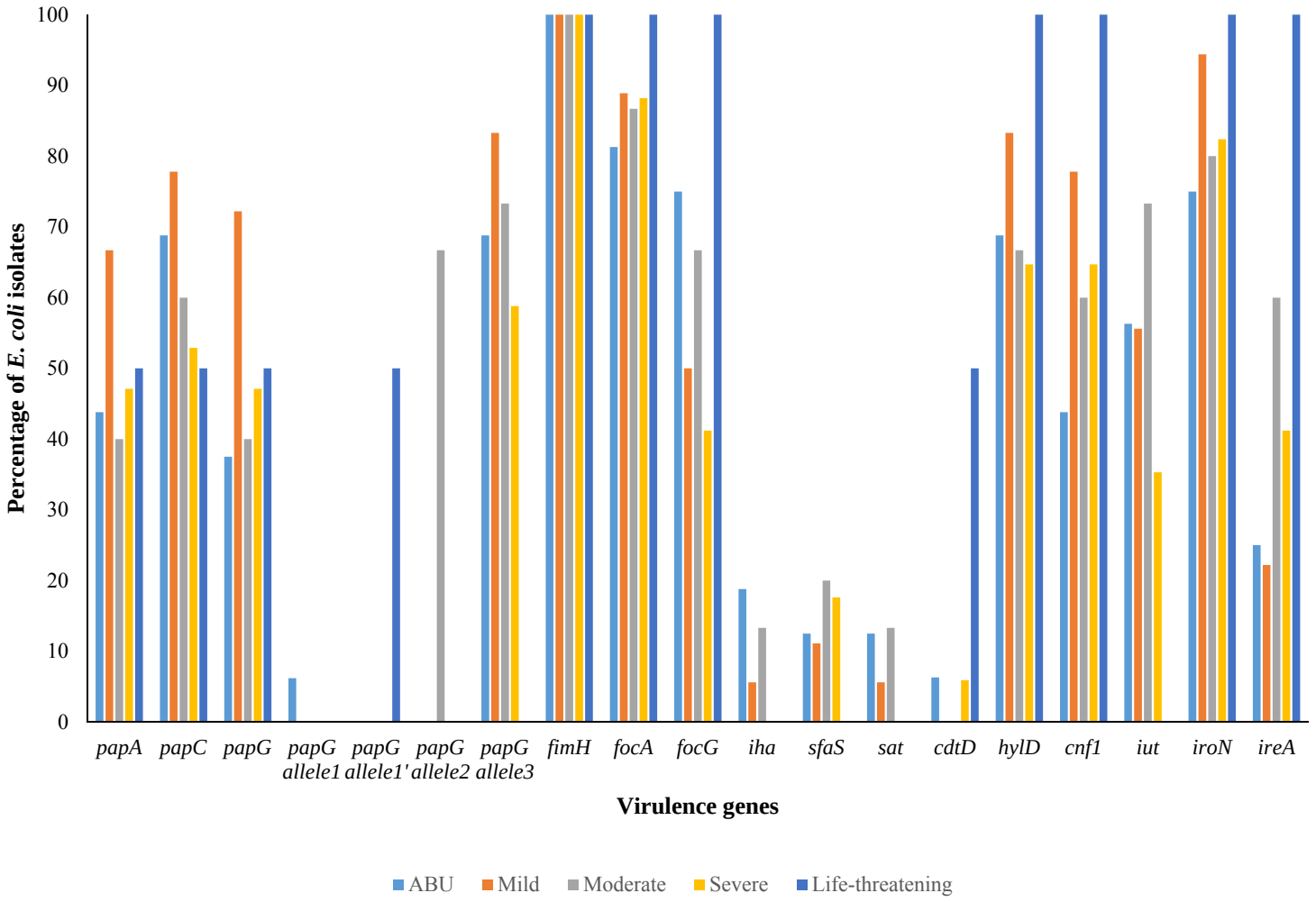

Figure 4.3 The proportion of 14 different virulence genes (axis X) detected in *E. coli* isolates associated with different levels of clinical signs of UTI.

VF genes	Phylogenetic groups, No (%)			
	A (N=4)	**B1 (N=4)**	**B2 (N=7)**	**D (N=53)**
papA	1 (25.0)	0	6 (85.7)[a]	28 (52.8)
papC	1 (25.0)	2 (50.0)	7 (100)[a]	34 (64.2)
papG	1 (25.0)	0	7 (100)[a]	27 (50.9)
papG allele 1	0	0	1 (14.3)	0
papG allele 1'	0	0	0	1 (1.9)
papG allele2	0	0	0	1 (1.9)
papG allele3	2 (50.0)	2 (50.0)	6 (85.7)[a]	37 (69.8)
fimH	4 (100)	4 (100)	7 (100)	53 (100)
focA	3 (75.0)	3 (75.0)	7 (100)	46 (86.8)
focG	3 (75.0)*	4 (100)	3 (42.9)	31 (58.5)
iha	0	0	0	5 (9.4)
sfaS	0	0	0	9 (17.0)
sat	0	0	0	5 (9.4)
cdtD	0	0	0	3 (5.7)
hlyD	2 (50.0)	1 (25.0)	7 (100)[a]	39 (73.6)
cnf1	2 (50.0)	0	6 (85.7)[a]	35 (66.0)
iut	2 (50.0)	2 (50.0)	5 (71.4)	27 (50.9)
iroN	2 (50.0)	100	7 (100)[a]	44 (83.0)
ireA	1 (25.0)	1 (25.0)	2 (28.6)	20 (37.7)

Table 4.4 Distribution of VF genes in 68 *E. coli* isolates categorized by each of 4 phylogenetic groups—A, B1, B2, and D.

[a] VF genes prevalence was significantly higher than those in other groups ($P<0.05$).

While the proportion of each of the 14 virulence genes did not different among the 5 levels of severity (*P>0.05;* Table 4.2), the proportion did vary when isolates were categorized as either ABU vs non-ABU. Those genes present in a greater proportion of non-ABU isolates were: *papG, focG, cnf1,* and *ireA* (*P<0.05*) (Table 4.2). The best discriminator among these VF between ABU and non-ABU was *cnf1* (cytonecrotic factor) which was present in 44% of ABU VS 69% non-ABU (*P<0.05*).

Table 4.4 shows the relative frequency VF genes within strains belonging to the 4 phylogenetic groups. Isolates belonging to group A carried *focG*, the gene encoding F1C fimbrial adhesin, significantly more frequently than did strains of the other phylogenetic groups. The genes for a number of VF including *pap*, *hlyD, cnf1,* and *iroN* were significantly more frequent in strains belonging to group B2 (table 4.4).

4.3.4 Screening of clone O25b:H4-ST131

191 isolates were screened by used of a triplex PCR assay for clone O25b:H4-ST131. From these number, 3 isolates were detected as ST131, giving the prevalence 1.6%. These 3 isolates were resistant to ampicillin, doxycycline, cephalothin, cepodoxime, enrofloxacin and ciprofloxacin. Severity of these 3 isolates were severe urinary tract infections.

4.4 Discussion

This study demonstrates that canine *E. coli* from the urinary tract, expressing different levels of clinical signs, exhibit the distinctive virulence genotype patterns and phylogenetic background.

Identification of *E. coli* phylogenetic group as A, B1, B2 or D has proven useful in the identification of pathologic strains [16] [11]. Our finding demonstrated that the most common phylogenetic groups of UPEC were D (40%) and B2 (31%). Most notably, the proportion of group B2 was high for severe disease, while group D was high in ABU isolates. Consistently in human medicine, most UPEC in humans belong to phylogenetic group B2 (generally containing more virulence genes) and to a lesser extent group D (fewer genes) [22] [23, 24] [25]. In contrast, commensal and intestinal *E. coli* largely belong to group A and B1 [26] [27]. In veterinary practice, canine *E. coli* pathogens associated with UTI and pyometra have been derived from specific clones of the phylogenetic group B2 [28]. Consistently, several studies indicate that phylogenetic group B2 and D are pathogenic *E. coli*. Interestingly, this study demonstrated that ABU *E. coli* strains primarily belonged to phylogenetic group D which indicated the most evolutionary distance from group A. Also, the number of virulence genes in phylogenetic group D are less than those in group B2 [22].

Our findings showed that MDR isolates primarily belonged to group A and B1. Likewise, a study of fluoroquinolone-resistant UPEC isolates from human showed that the isolates belonged to non-virulent, commensal, phylogenetic groups A or B1 [17]. Moreover, extended-spectrum beta-lactamases producing *E. coli* isolates from chickens and swine also primarily belonged to phylogenetic groups A, D, and B1. Analysis of phylogenetic background, pathogenicity islands, and antimicrobial resistance of *E. coli* isolates from human and animal wastewater revealed that in both groups, *E. coli* from A, B1, and D groups were prevalent, and strains from both origins showed a similar pattern of virulence genes in each phylogenetic group.

In the present study, the isolates were screened for presence of the 14 virulence genes. Virulence profiles of canine and feline UPEC were different in the levels of severity; however, no one specific gene studied thus far was able to predict the difference of severity. This data supports findings of previous investigators that have demonstrated the importance of multiple virulence factors in the role of infection or localization of *E. coli* colonizing or infecting extraintestinal environments. Notably, although virulence genes may be present in ABU isolates, they may not be expressed, or if expressed, the protein may be non-functional. Accordingly, our attempt at identifying a predictive model for pathogenic UPEC must also focus on gene expression of these virulence genes (see chapter 5).

Our findings reported that the high prevalence of *papG* allele III (69%) in canine UPEC is irrelevant for *E. coli* isolates from human source. PapG expresses different binding specificities to different receptor isotypes including I, II, and III [29], so that canine UPEC might express PapG to specific canine uroepithelium. It corresponds with the early observation by Marklund et al. that *pap*-positive canine fecal *E. coli* isolates contained only *papG* allele III and with the recent demonstration of *papG* allele III as the most prevalent *papG* allele within a small set of canine urine and fecal isolates [30]. Moreover, Marklund et al. found only the *papG* allele II among their human-source controls and suggested that *papG* allele III is specific to dogs while that *papG* allele II is specific to humans [30]. It is now known, however, that *papG* allele III was found among *E. coli* isolates from human cystitis [31]. Thus, the statement that *pap*-positive canine strains (allele III) are irrelevant for human disease is not correct.

In recent years, the worldwide dissemination of antimicrobial resistant *E. coli* clones of specific sequence types has become a concern in epidemiology. A previous study

demonstrated that the O25b:H4-ST131 was found in 17% of population from patients hospitalized in 33 widely distributed US medical centers [25], and was present 5.6% of 177 phenotypically ESBL-producing *E. coli* isolates among companion animals in US [32]. This study has reported 3 isolates of 191 isolates (1.6%). The prevalence of O25b:H4-ST131 was inconsistent to previous reports; this might reflect a different population of isolates between the studies (ESBL-producing, or fluoroquinolone resistant *E. coli* isolates). Further investigation would help to understand the prevalence of these sequence types clones among companion animals.

4.5 Conclusion

In summary, we found that canine *E. coli* from urinary tract expressed different levels of clinical signs, exhibited the distinctive patterns of association with virulence genotype and phylogenetic background. Mostly, *E. coli* isolates with UTI were phylogenetic groups B2, and D. However, the MDR *E. coli* isolates was mostly belong to A and B1 which was associated with marked reductions in inferred virulence. This suggests that in *E. coli* the relationship between antimicrobial resistance and virulence varies according to resistance phenotype, with MDR usually occurring in intrinsically low-virulence non-B2 or D strains rather than via loss of VFs from virulent strains. This study suggested that identification of phylogenetic group and virulence genes of *E. coli* may help in the pre-treatment identification of ABU *E. coli.*

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CHAPTER 5

PREDICTING ASYMPTOMATIC BACTERIURIA IN CLINICAL *ESCHERICHIA COLI* UROPATHOGENS

5.1 Introduction

E. coli are the predominant cause of UTI in both human and small animals. Pathophysiology of UTI involves ascending translocation of microorganisms from the gastrointestinal tract. However, to translocate, these organisms must acquire virulence factor (VF) genes that facilitate extraintestinal survival [1]. In the process of survival, UPEC cause signs indicative of UTI in the host. Different VFs lead to differences in the extent and impact of infection and thus variable clinical signs, including lack of symptoms (asymptomatic bacteriuria [ABU]), mild, moderate, severe (pyelonephritis), and life-threatening (pyelonephritis, or urosepsis). UPEC pathogenesis begins with bacterial adhesion (*pap, fim, focA*) to uroepithelial cells. Toxins (e.g, cytotoxic necrotizing factor [*cnf*], hemolysins [*hyl*]) damage outer cells, and iron scavenging mediated via siderophores *(ireA*), facilitates penetration. Clinical signs associated with UTI presumably vary with the virulence genes expressed by the colonizing or infecting microbe [2].

ABU is defined as a significant number of bacteria in the urine without the usual symptoms of urinary tract infection [3]. The pattern of VF gene expression in UPEC might vary with the different severities of disease. ABU isolates appear to act as if commensal in the bladder, out-competing other microbes, and potentially protecting the bladder from colonization by more pathogenic strains. Regardless, antimicrobial treatment of ABU isolates may not be prudent: not only is the risk of multidrug antimicrobial resistance increased [4], but their removal may allow infection of the bladder with more pathogenic organisms. Convincing practitioners to

not treat ABU with antimicrobials requires evidence that *E. coli* associated with ABU lack or do not express genes typical of UPEC. This study proposes to identify gene expression that will discriminate canine ABU *E. coli* from UPEC.

Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set [5]. It accomplishes this reduction by identifying directions, called principal components (PC), along which the variation in the data is maximal [6]. By using a few components, each sample can be represented by relatively few numbers instead of by values for hundreds of variables. Samples can then be plotted, making it possible to visually assess similarities and differences between samples and determine whether samples can be grouped. PCA has previously been demonstrated to be a good method for analysis gene expression [7]. UPEC are classified as different severity (ABU, mild, moderate, severe, life-threatening), virulence genes expression is known to correlate with different severities. The goal of this study is to characterize canine UPEC in order to classify the severity of UTI and to identify potential ABU isolates that might be developed as a biotherapeutic alternative to antimicrobial therapy in the dog or cat.

5.2 Materials and methods

5.2.1 *E. coli* isolates

A total 68 canine UPEC isolates were randomly selected from our library of 3172 isolates from the private small animal hospitals. Based on the submitting practitioners' assessment, each isolate was categorized as to severity of clinical signs: none (ABU; n=15), mild (n=18), moderate (n=15), or severe (n=20) and combined as a fifth category, non-ABU (n=53).

5.2.2 RNA purification and reverse transcription

The bacterial isolates were grown in Luria-Bertani (LB) (Difco Laboratories, Detroit, MI) broth and incubated at 200 rpm at 37ºC until the OD_{600} of 0.7–0.8 corresponding to the mid-logarithmic phase, and followed by RNA extraction using Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA concentrations and A260/A280 were measured by using a Nanodrop 2000® Spectrophotometer (Thermo Scientific, Wilmington, DE). *E. coli* total RNA 1 µg was reverse transcribed with 100 U of the iScript™ cDNA Syntheses Kit (Bio-Rad Laboratories, Inc., Valencia, CA). Reverse transcription included 30 min incubation at 25°C followed by a synthesis step of cDNA proceeded for 30 min at 42°C and another 5 min at 85°C using a commercial enzyme and iScript™ cDNA Syntheses Kit (Bio-Rad Laboratories, Inc., Valencia, CA).

5.2.3 *q*RT-PCR experiments

Primers (Table 5.1) were selected from genetic codes in GenBank and previous study (Johnson et al., 2008). *q*RT-PCR was performed using Roche® DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN) in the Roche® Light-Cycler 480. *q*RT-PCR was used to assess the expression level of RNA coding for the 7 VF genes and one housekeeping gene (*gapA*). Seven VF genes were categorized as to adhesins (*pap, focG, fimH,* and *focA*), toxins (*hlyD,* and *cnf1*), or iron acquisition proteins (*ireA*). Primers of virulence genes were used as described previous study (chapter 4). *q*RT-PCR conditions included an initial denaturation at 95°C for 10 min followed by 45 amplification cycles (95°C for 10 s, 61°C for 20 s and 72°C for 29 min).

5.2.4 Data calculations and interpretations

The relative expression of 7 VF genes was calculated by the level of relative quantification in each gene divided by that of *gapA* gene, a housekeeping gene. After *q*RT-PCR,

the number of PCR cycles to reach the fluorescence threshold in each sample was defined as the cycle threshold (CT). To moderate the level of gene expression, the natural log transformation was applied to all ratios (Eisen et al., 1998). CT values of 7 VF genes were directly related, since the input of cDNA was equal for each PCR reaction. Normalized CT values followed this formula:

(Formula 5.1)

$$\text{Level of gene expression} = \text{Ln}(CT_{sample}/CT_{control})$$

$$= \text{Ln}(CT_{VF\ gene}/CT_{gapA})$$

$$= \text{Ln}(CT_{VF\ gene}) - \text{Ln}(CT_{gapA})$$

5.2.5 Statistical analyses

The original data was log-transformed and normalized with the *gapA* gene before to further analysis. Since some of these VF genes are presumably related each other; notice from their functions, the levels of their expression might have correlations. All analyses were using the SAS package (SAS Institute Inc.).

a) Principal component analysis

The PCA was used to reduce used for reduction the 7 variables of gene expression to a fewer PCs and identify which genes are the most contributor in each component. A matrix of expression data was created, *A*, where each row corresponds to a different isolate and each column corresponds to one of different VF genes. The a_{ij} entry of the matrix contains the j^{th} gene's relative expression ratio with respect to a control *gapA* gene. To compute the principal components, the *n* eigenvalues and their corresponding eigenvectors are calculated from the *n*x*n* covariance matrix of isolates. Each eigenvector defines a PC. A PC can be viewed as a weighted

sum of the isolates, where the coefficients of the eigenvectors are the weights. The projection of gene *i* along the axis defined by the j^{th} PC is:

$$a'_{ij} = \sum_{i=1}^{n} a_{it} v_{tj}$$

Where v_{tj} is the t^{th} coefficient for the j^{th} PC; a_{it} is the expression measurement for gene *i* from each isolate. *A'* is the data in terms of PCs. Since *V* is an orthonormal matrix, *A'* is a rotation of the data from original space of observations to a new space with PC axes.

The variance accounted for by each of the components is its associated eigenvalue; it is the variance of a component over all genes. Consequently, the eigenvectors with large eigenvalues are the ones that contain most of the information; eigenvectors with small eigenvalues are uninformative.

b) Linear discriminant function analysis

To cluster for each severity of clinical signs, linear discriminant function analysis (LDA) performs a multivariate test of differences between groups, and constructed LDA classifiers (nearest centroid classifiers). LDA used all 4 PCs when computing the standardized distance to each centroid.

In the analysis of the expression of different VF genes, LDA was implemented for clustering with a categorical target variable which, in this study, is 4 different severities of disease or two categories of ABU and non-ABU. The first four PCs were used as the variables. Cross tabulate computation was used to calculate the error rate of the prediction.

5.3 Results

5.3.1 Principal component analysis

The data contains expression ratios for seven VF genes from *E. coli* isolates. The data was collected for each gene of 68 *E. coli* isolates. Thus, the matrix to be analyzed has 68 rows of isolates and 7 columns of genes corresponding to each of measured points. Table 5.1 reports the mean, and standard deviation of each gene from overall isolates.

VF genes	Mean±SD (n=68) Original data	Mean±SD (n=68) Natural log transform
fimH	2.9±13.5	-1.9±3.1
focA	11.2±9.0	-1.4±3.4
ireA	42338.0±22054	2.9±0.6
papC	25.6±15.4	-0.6±0.4
papG	316857.9±311610	2.7±0.5
hlyD	7.1±4.0	-0.4±0.2
cnf1	1.5±0.2	-0.3±0.2

Table 5.1 Summary of the data collected from *q*RT-PCR and natural log transform. The table contains mean relative expression ratios to *gapA* gene of both original and transformed data. (SD = standard deviation).

Table 5.2 contains all 7 PCs and their corresponding eigenvalues. Figure 5.1 is a plot of the eigenvalues of the components. Four eigenvalues lie above the 80% cutoff, suggesting two dimensions for the VF gene expression data. The first four PC account for over 85% of the total

variability; inclusion of the fifth PC increases this to almost 95%. The meaning of these PC can be distilled from their respective coefficients. The first PC represents a weighted average and distinguishes genes by their average overall expression. The coefficients are proportional to the variance of the each gene that are associated with it. The correlation matrix of gene expression values demonstrated that these 7 genes were not highly correlated (correlation=0.3) and none of correlation values among these 7 genes were more than 0.4. Functionally, virulence genes were related to each other for colonization in host cells, so that the PCA was still able to reduce this this study to fewer components.

VF gene	**Coefficients of the PCs**						
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
fimH	-0.24	-0.21	-0.16	0.48	0.79	-0.23	0.10
focA	0.35	-0.08	0.18	-0.72	0.50	-0.26	-0.06
ireA	0.85	0.34	0.07	0.39	0.04	0.05	0.11
papC	-0.04	-0.38	0.88	0.26	-0.01	-0.05	-0.02
papG	-0.33	0.83	0.39	-0.13	0.10	0.10	0.10
hlyD	0.02	-0.17	0.01	-0.08	0.32	0.92	0.08
cnf1	-0.02	-0.12	-0.01	-0.11	-0.09	-0.09	0.98
Eigenvalue	30.4	19.7	12.0	10.4	7.7	2.2	1.4
% variance	37%	24%	14%	12%	9%	3%	1%
% cumulative variance	37%	61%	75%	87%	96%	99%	100%

Table 5.2 PCA was implemented to create a model based on VF expression that predicts clusters of UTI clinical signs. The values in the column are coefficients of the PCs that are related to each of VF gene. For the first component, *ireA* was the greatest contributor to variance, with a 0.85 coefficient value, followed by *papG* (2nd PC), with 0.83. The 3rd PC was *papC* with 0.88 and the 4th PC was *focA* with 0.72. The eigenvalues express the variance of a PC overall genes. PC1-PC4 contain over 80% of the total variance in the data.

The first PC is an average expression weighted by the information content (i.e. variance) of a particular experiment, and the *ireA* gene was the greater contributor, with 0.85 coefficient value in this component. One pattern in the second PC of expression was represented by *papG*, and the third PC encompassed *papC*, with a similar coefficient value at approximately 0.8. The other genes, in the first four components, contributed a weight of no more than 0.5 coefficient values per gene. Therefore, each PC had only one gene contribute to each component.

The first four PCs explained 87% of total variability (Table 5.2). The addition of fifth PCs was increased only 9% of total variability so that the first 4 PCs were considered to further analysis.

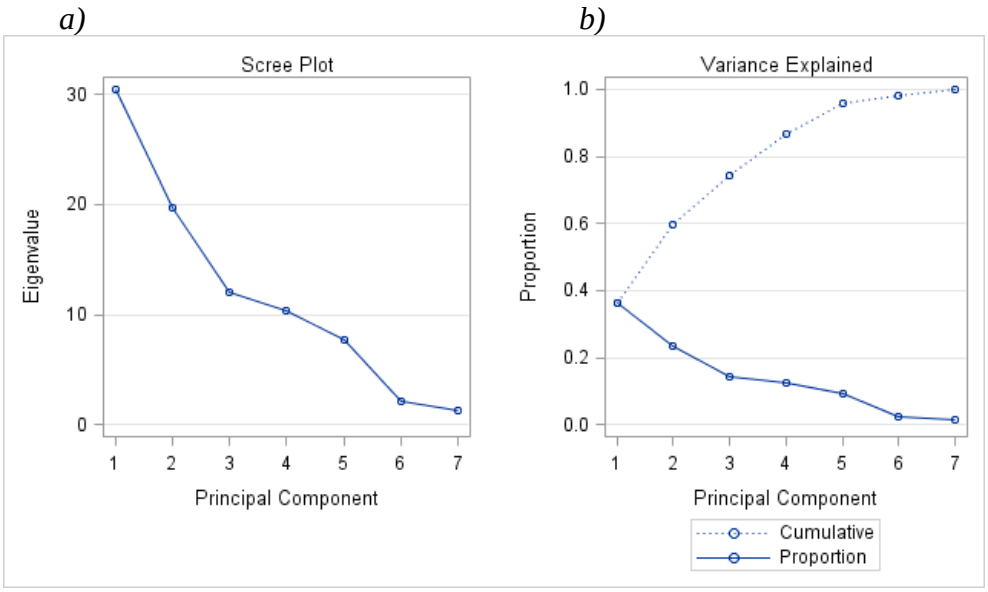

Figure 5.1 *a)* Plot of eigenvalues of the PC. *b)* Plot of % variance explaining the percent of total variance. Most of the variance in the relative expression ratio data set is contained in the first four PCs.

5.3.2 Linear Discriminant Analysis

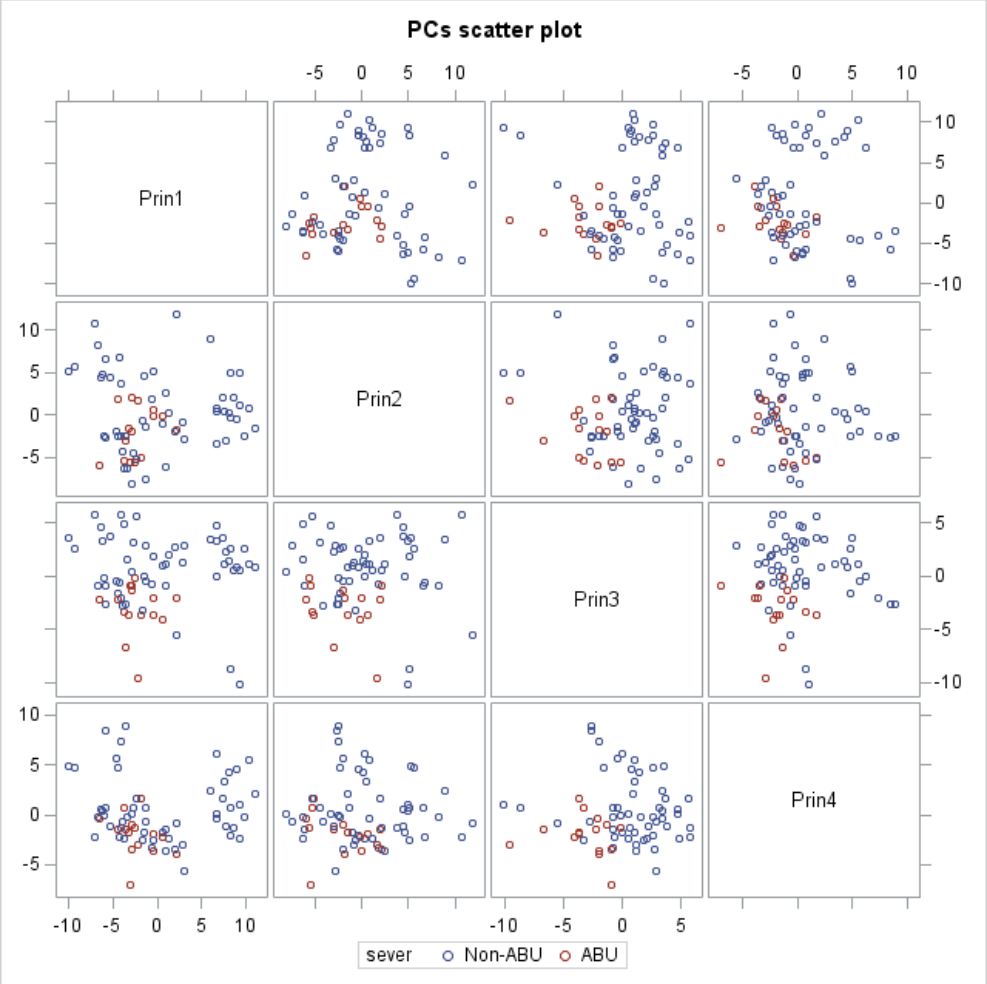

Figure 5.2 Matrices of scatter plots among the 4 PCs visualized after clustering based on 2 groups, (ABU and non-ABU) of clinical signs of UTI. PCs are new variables generated from combination of related genes. Relatedness of the genes in each PC is based on the grouping that best explains the variability of gene expression.

To cluster the data, LDA was implemented to classify the different severities of disease based on a linear model with the 4 PCs. The cross-validation results (table 5.3) showed a test accuracy rate 43/68 (63.2%) or error rate 25/68 (36.8%), using 4 PCs. However, in ABU group, this model computed an error rate 1/15 (6.7%).

Severity	ABU	Mild	Moderate	Severe	Total
Actual (n=)	15	18	15	20	68
Predicted (n=)	14	7	10	12	43
Accuracy rate (%)	93.3	38.9	66.7	60	63.2
Error rate (%)	6.7	61.1	33.3	40	36.8

Table 5.3 PC1-PC4 were subjected to linear discriminant function to predict the isolates as to the four severities of clinical signs. Cross validation from the predicted or classification from model and observed value from questionnaires demonstrated a high error rate (>50%) and low prediction rate (<50%).

Since the observations of severities were obtained from different clinicians, the differentiation of severity may different. Thus, limiting categories to ABU versus non-ABU might be more appropriate for this analysis. Accordingly, LDA was repeated based on two classifications: non-ABU and ABU. The cross-validation of classification results were summarized in table 5.4. The discrimination provided the satisfied error of estimation with value 9.4%. The correct estimation rate was 90%. In addition, this model computed a classification of ABU with 0% false negative.

Severity	Non-ABU	ABU	Total
Actual (n=)	43	15	58
Predicted (n=)	53	15	68
Accuracy rate (%)	81.1	100	85.3
Error rate (%)	18.9	0	14.7

Table 5.4 PC1-PC4 were subjected to linear discriminant function to predict the isolates as either ABU or non-ABU. Cross-validation from the prediction from model and observed value from questionnaires demonstrated 0% of negative false rate and low as 19% of false positive rate prediction.

5.4 Discussion

To characterize in more detail the virulence of UPEC, the expression of 7 VF genes was investigated in 68 canine UPEC. Gene expression profiles of UPEC have been studied previously [8], but not based on classification of severity of clinical signs. This is the first study to characterize VF gene expression of canine UPEC with the intent to classify the severity of clinical signs. Our results with VF gene expression confirm that PCA can find a reduced set of variables from 7 virulence genes to 4 PCs. Also, this study is the first to demonstrate distinguishing different severity of UTIs based on expression of virulence genes.

PCA has been used in this study for multivariate analysis to reduce the dimensions of data set, and to develop a diagnosis for ABU. PCA is often used as a preprocessing step to clustering [9]. This analysis can be considered useful tools to investigate correlation patterns among the numerous measured variables. Since VF is of recognized importance in the pathogenesis of UTI include adhesion, siderophores, toxin and others, and their expression

correlates with severity [1], their levels of expression reasonably might predict the severity of clinical signs. PCA might be used to understand the complex processes of virulence gene expression [7].

To discriminate the levels of genes' expression by different severities of disease, the LDA model was computed. This model estimated the 4 different severities of disease with the high error rate. However, LDA that classified 2 groups as ABU and non-ABU provided the better error rate (19%) and 0% of false negative. Thus, this study suggests that a model that can predict different levels of severities in practices still need to further develop.

These results suggest that further addition of more VF genes expression can be identified the different severities of disease may help in the pre-treatment identification of ABU *E. coli* such that a "no-antimicrobial treatment" option might be considered. Further, such isolates might be developed as a therapy alternative to antimicrobials. Both goals will support de-escalation of antimicrobial use and thus antimicrobial resistance.

5.4 Conclusion

Virulence profiles of clinical canine UPEC could not accurately predict differences between ABU and non-ABU unless subjected to PC analysis. The success of PCA in discriminating ABU vs non-ABU suggests that further clustering analysis of VF may identify a VF profile that discriminates more discrete categories of UTI severities. This can be useful in a clinical setting by helping identify those isolates that do not have to be treated with antimicrobial drugs. Further, ABU isolates might be developed as a therapeutic alternative to antimicrobial therapy. Both goals will support de-escalation of antimicrobial use and thus antimicrobial resistance.

5.5 References

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CHAPTER 6

GENERAL CONCLUSION

Since the incidence of antimicrobial resistance had been rapidly increasing, it has become important to reduce the inappropriate use of antimicrobials and also to optimize the dosing regimens to prevent the development of resistance. These studies were designed to establish the current status of *E. coli* susceptibility to routinely-selected antimicrobials and to assess veterinary antimicrobial prescribing behaviors as they relate to judicious antimicrobial by national and regional levels.

This study demonstrates that clinical *E. coli* isolates collected from dogs and cats express resistance to at least one drug studied here, with high level resistance of commonly-used first tier antimicrobials. Generally, clinicians use empirical antimicrobial therapy and a variety of dosing regimens. Thus, their selection and dosing regimen to treat *E. coli* infection in dogs and cats may be ill advised. Improving the procedures of antimicrobial prescription may possibly lead to decrease the therapeutic failure and prevalence of emerging antimicrobial resistance.

The relationship between antimicrobial resistance and virulence varies according to resistance phenotype, with MDR usually occurring in intrinsically low-virulence non-B2 or D strains rather than loss of VFs from virulent strains. Virulence profiles of clinical canine UPEC could not accurately predict difference between ABU and non-ABU but PC analysis suggests that further clustering analysis of VF may identify a VF profile that discriminates between ABU and non-ABU prior to treatment. These studies provided guidance regarding the need for antimicrobial treatment of ABU *E. coli*-including diagnostic tests to detect ABU.