IMPACT OF ROUTINE ANTIMICROBIAL THERAPY ON
FECAL Escherichia coli IN DOGS

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IMPACT OF ROUTINE ANTIMICROBIAL THERAPY ON

Fecal *Escherichia coli* in Dogs

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IMPACT OF ROUTINE ANTIMICROBIAL THERAPY ON

FECAL *Escherichia coli* IN DOGS

Nipattra Debavalya

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Antimicrobial resistance is an emerging aspect of antimicrobial therapy. However, few studies have documented this relationship in vivo. The purpose of this study was to describe the relationship between antimicrobial therapy and emerging antimicrobial resistance within the normal flora. The dog was chosen as the model because it is the target species to which therapy is directed and it has a close relationship to humans. Fecal \textit{E. coli} were chosen as the sentinel organism because of ease of access, the fast reproductive time, high rate of mutability and it is the common cause of disease, particularly urogenital, in dogs and humans. Amoxicillin and enrofloxacin were chosen
as the target drugs because they are common choices for treatment of *E. coli* associated disease and different resistance mechanisms were anticipated.

We hypothesized that amoxicillin resistance would be short-lived, oriented toward beta-lactams and plasmid mediated, whereas enrofloxacin resistance would persist, but be limited to enrofloxacin. Amoxicillin or enrofloxacin was administered to 8 healthy, antimicrobial-free purpose-bred dogs; no drug was administered to 8 control dogs until resistance was expressed in their fecal *E. coli*. The drug was then discontinued and monitoring continued until resistance was either resolved or 28 days had passed. Representative bacterial isolates expressing resistance were collected from each dog per group per time point. Each isolate was serotyped and tested for virulence. Each isolate was characterized for the degree of resistance and whether the resistance was to multiple drugs. In addition, representative isolates from each group were serotyped.

Close to 100% of fecal *E. coli* isolates rapidly became resistant to both drugs. Amoxicillin resistance resolved in 7-9 days. In contrast, all *E. coli* were eradicated in 4 dogs receiving enrofloxacin. In the remaining dogs, resistance resolved in 11-21 days but did not resolve in one dog by the end of the study. Resistance to both drugs was at least 16 fold higher than the breakpoint (high level). Resistance associated with enrofloxacin but generally not amoxicillin was multidrug resistance (MDR). Amoxicillin therapy induced resistance to penicillins, selected cephalosporins but not carbapenems. Beta-lactamase resistance was due to TEM β-lactamase (TEM). It was horizontally transferred, with the exception of extended-spectrum β-lactamase enzymes (ESBLs), which were detected only in selected isolates. Amoxicillin therapy induced variable phenotypes and genotypes. One dog receiving amoxicillin developed MDR (including
enrofloxacin) resistance. For enrofloxacin treated dogs, genotypes within phenotypes were less variable. All isolates expressed TEM β-lactamase mediated by a non-transferrable mechanism. Resistance to fluoroquinolone (FQ) was mediated by double mutations in both gyrA and parC. Mutation in a global regulator SoxS was found and our data indicate that this oxidative stress response regulator may impact MDR mediated by enrofloxacin in fecal E. coli. Among serotypes, more variety occurred in non-MDR than in MDR or in control dogs, with clonality associated with enrofloxacin but not amoxicillin resistance. Virulence factors were limited to cnf-1 and cnf-2 which were detected in only 5 isolates.

In conclusion, these studies indicated that a high level of antimicrobial resistance rapidly evolved in E. coli from dogs treated with either amoxicillin or enrofloxacin. However, resistance resolved more quickly with amoxicillin, and was associated with multiple drugs for enrofloxacin. Non-ESBL beta-lactamase resistance occurred only with amoxicillin and was transmissible. We report for the first time (1) a substitution of alanine for glutamate at codon 84 of parC gene is associated with enrofloxacin in E. coli and (2) a point mutation leading to a substitution of serine for alanine at codon 12 of a general regulator soxS in MDR is associated with enrofloxacin therapy.
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Computer software used  **MICROSOFT WORD 2003**
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CfU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum beta-lactamase</td>
</tr>
<tr>
<td>FQ</td>
<td>Fluoroquinolone</td>
</tr>
<tr>
<td>H</td>
<td>Hour</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;BP&lt;/sub&gt;</td>
<td>Breakpoint MIC</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse-field gel electrophoresis</td>
</tr>
<tr>
<td>q</td>
<td>Every</td>
</tr>
<tr>
<td>REC</td>
<td>Resistant <em>E. coli</em></td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>T1/2</td>
<td>Elimination half-life</td>
</tr>
<tr>
<td>TCC</td>
<td>Total coliform counts</td>
</tr>
<tr>
<td>TEC</td>
<td>Total <em>E. coli</em> counts</td>
</tr>
<tr>
<td>TRCC</td>
<td>Total resistant coliform counts</td>
</tr>
<tr>
<td>TREC</td>
<td>Total resistant <em>E. coli</em> counts</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
</tbody>
</table>
1.1 Antibiotics, antibacterial and antimicrobial drugs

The scientific use of the word “Antibiosis” was first introduced by the French biologist Jean-Paul Vuillemin in 1889 to denote destruction of one organism by another.1 As noun, “Antibiotics” was described by the Nobel laureate microbiologist Selman A. Waksman in 1942 as “chemical substances that are produced by microorganisms and that have the capacity, in dilute solution, to selectively inhibit the growth of and even to destroy other microorganisms”.1-3 The concept of antibiosis started when Alexander Fleming discovered in 1929 that the colonies of Staphylococcus sp. were lysed on a plate contaminated with a penicillium mold. Few years later, Chain, Florey and associates (1940) purified and synthesized penicillins from Penicillium notatum cultures.2,4

An antimicrobial agent, with a broader definition, is defined as a substance of natural, semisynthetic, or synthetic origin that kills or inhibits the growth of a microorganism but causes little or no damage to the host.1-2, 4 As such, antimicrobial drugs can be either antibacterial or antifungal.2,4 However, in many publications nowadays, the term “antibiotics” is commonly applied to all drugs capable of inhibiting
bacterial growth, as synonymous or interchangeable with antibacterial or antimicrobial drugs.²⁻⁴

1.2 Classification of antimicrobial drugs

Antimicrobial drugs are characterized by various criteria such as chemical structure, spectrum of activity and mechanism of action (Table 1-1).²⁻³ 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 from the core structure leads to various members of the class and influences their spectrum of activity, pharmacodynamics, and toxicology.¹⁻³

On the basis of range of susceptible bacterial group or spectrum of activity, antimicrobials are classified as broad-, intermediate- and narrow-spectrum. Broad-spectrum antimicrobial agents such as tetracycline, carbapenems, and third and fourth generation cephlosporins are effective against wide variety of bacteria species, including Gram positive organisms, Gram negative organisms, aerobes and anaerobes. Narrow-spectrum antimicrobials, on the other hand, are mainly active against a specific bacterial group like Gram positive organisms (e.g., natural penicillins, bacitracin), Gram negative (e.g., polymyxins), aerobes (e.g., aminoglycosides, sulfonamides), or anaerobes (e.g., nitroimidazoles).¹⁻²
Additionally, antimicrobials are also grouped by their ability to inhibit or kill bacteria. Some drugs inhibit growth of a bacterium at one concentration [minimal inhibitory concentration (MIC)] but require a higher concentration to kill it [minimum bactericidal concentration (MBC)]. An antibacterial drug that exhibits a large difference between MIC and MBC is considered as a bacteriostatic drug, while an antibacterial that kills a bacterium at or near the same concentration that inhibits its growth is considered as a bactericidal drug.2 This classification is also dependent on how drugs bind to targets. Antimicrobial drugs that kill bacteria are termed “bactericidal” and these drugs bind targets irreversibly or with high affinity. Contrarily, drugs that inhibit or delay bacterial growth are called “bacteriostatic” and these drugs usually form unstable bonds with targets.1 However, this classification is quite arbitrary, as some drugs may exhibit either bactericidal or bacteriostatic effects depending on the drug concentration, type, quantity, growth state of bacterium and the experimental conditions for which the drug activity is evaluated.1-2 In clinical practice, in contrast, this classification is an important distinction. Since bacteriostatic drugs have slower effects that are dependent upon host immune response to kill and eliminate bacteria, they are not normally recommended for immunosuppressed patients or treatment of life-threatening acute infections like meningitis.1

Based on mechanism (sites) of action, antimicrobial drugs are generally divided into 4 main categories; (1) Inhibition of cell wall synthesis, (2) Inhibition of protein synthesis, (3) Inhibition of nucleic acid synthesis, and (4) Inhibition of folic acid biosynthesis (Figure 1-1).1-5
Table 1-1 Classes of antimicrobial drugs

<table>
<thead>
<tr>
<th>Class</th>
<th>Drugs</th>
<th>Spectrum of activity</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-lactams</strong> (penicillins, cephalosporins, carbapenems, monobactams)</td>
<td>Penicillin, Ampicillin, Amoxicillin, Cephalothin, Imipenem</td>
<td>Bactericidal against Gram positive and/or Gram negative bacteria</td>
<td>Inhibit cell wall synthesis</td>
</tr>
<tr>
<td></td>
<td>Aminoglycosides</td>
<td>Gentamicin, Amikacin, Streptomycin</td>
<td>Broadly bactericidal</td>
</tr>
<tr>
<td></td>
<td>Tetracyclines</td>
<td>Tetracycline, Doxycycline</td>
<td>Broadly bacteriostatic; some protozoa</td>
</tr>
<tr>
<td></td>
<td>Macrolides</td>
<td>Erythromycin, Azithromycin</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td></td>
<td>Fluoroquinolones</td>
<td>Norfloxacin, Ciprofloxacin, Enrofloxacin</td>
<td>Broadly bactericidal; can enter phagocytes, kill intracellular bacteria</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim/sulfonamides</td>
<td>Trimethoprim/sulfamethoxazole</td>
<td>Broad antibacterial; some fungi, protozoa</td>
</tr>
<tr>
<td></td>
<td>Phenicols</td>
<td>Chloramphenicol</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td></td>
<td>Glycopeptides</td>
<td>Vancomycin</td>
<td>Bactericidal; most effective against Gram positive bacteria</td>
</tr>
<tr>
<td></td>
<td>Lincosamides</td>
<td>Lincomycin, Clindamycin</td>
<td>Bactericidal</td>
</tr>
</tbody>
</table>
1.2.1 Inhibition of cell wall synthesis

The cell wall is an essential component of bacterial cell. Peptidoglycan is an important part of cell wall structure in both Gram positive and Gram negative bacteria. In Gram positive bacteria, peptidoglycan is substantially thicker with multiple layers. However, Gram positive bacteria lack an outer membrane thus their thick peptidoglycan layer has been described as a surface organelle, for display of carbohydrates and proteins.
Gram negative bacteria, in contrast, have thinner peptidoglycan surrounded by an outer membrane, which is equivalent to a surface organelle in Gram positive bacteria.\textsuperscript{1, 4, 6}

However, most antibiotics do not work well against Gram negative bacteria because of the outer membrane through which substrates have to go through the small pores created by porins. Limited pore sizes of porin proteins reduce and prevent inward passage of antibiotics, like glycopeptides, into the periplasmic space.\textsuperscript{1, 6-7}

There are 3 main phases in peptidoglycan synthesis. The first phase is the cytoplasmic phase in which the structural units of peptidoglycan layers named N-acetyl glucosamine and N-acetyl muramyl pentapeptide are synthesized in cytoplasm. These subunits of peptidoglycans are bound to the cytoplasmic surface of the cell membrane and translocated externally by lipid carriers. Then in the last step, extracytoplasmic phase, glycan and peptide strands of muramyl pentapeptide are cross-linked by membrane-bound transglycosylases and transpeptidases respectively.\textsuperscript{8} Beta-lactams and glycopeptides inhibit cell wall synthesis at the extracytoplasmic phase while fosfomycin and bacitracin affect cytoplasmic phase and membrane-associated phase respectively.\textsuperscript{1, 4, 8-9}

1.2.2 Inhibition of protein synthesis

Proteins are important in bacterial life since enzymes and cellular structures are generally proteins. Protein synthesis is catalyzed by ribosomes. It starts with transcription of DNA into mRNA followed by mRNA translation and translocation. In translation,
which is the most frequently targeted by antibacterial drugs, bacterial ribosome reads mRNA and translates it into amino acid sequences.\textsuperscript{10}

Although mechanism of protein synthesis in prokaryotes and eukaryotes is similar, prokaryotic ribosomes differ from those in eukaryotes. This explains the effectiveness and selective toxicity of many antibacterial drugs. Bacterial ribosomes are composed of 2 subunits, a small 30S subunit containing 21 proteins and 16S rRNA, and a large 50S subunit containing 31 proteins, 23S and 5S rRNA. Some drugs, such as aminoglycosides, tetracyclines, target bacterial ribosome by binding to 16S rRNA of 30S subunit, whereas others such as chloramphenicol, macrolides and lincosamides bind to 23S rRNA of 50S subunit. Aminoglycosides bind to 30S ribosomal subunit irreversibly, leading to incorporation of incorrect amino acids, formation of nonfunctional initiation complexes and rapid cell death.\textsuperscript{1, 10-11}

1.2.3 Inhibition of nucleic acid synthesis

Nucleic acid synthesis is a vital function for the bacterial cells. DNA replication is a process by which genome is copied during cell division. Transcription is a process by which mRNA is synthesized from a DNA template, resulting in the transfer of genetic information from the DNA molecule to the messenger RNA. Translation is a process in which mRNA is converted into polypeptide by ribosomes. Fluoroquinolones inhibit bacterial DNA gyrase and topoisomerase IV enzymes, leading to inhibition of DNA
synthesis, whereas rifamycins such as rifampicin inhibit RNA polymerase to interrupt RNA synthesis.\textsuperscript{1,3,10}

### 1.2.4 Inhibition of folic acid biosynthesis

Sulfonamides and diaminopyrimidines (e.g., trimethoprim) have an indirect inhibitory effect on nucleic acid synthesis by blocking folic acid synthesis. Bacteria cannot utilize exogenous folic acid and therefore have to produce it. Sulfonamides are structurally similar to PABA (para-aminobenzoic acid), a first substrate in the pathway. Thus, they competitively bind to pteridine in the first step, inhibiting the action of dihydropteroate reductase enzyme in folic acid biosynthetic pathway. Trimethoprim resembles dihydrofolic acid and competitively inhibits dihydrofolate reductase enzyme that catalyzes the last step in the pathway.\textsuperscript{1,3,5}

### 1.3 Beta-lactam drugs

\(\beta\)-lactam drugs were first observed in 1929 when Alexander Fleming discovered penicillin. Later, in 1940, Chain and Florey extracted penicillin from \textit{penicillium\ notatum}.\textsuperscript{2,4,12} In 1945, cephalosporins were first isolated from \textit{Cephalosporium acremonium} (or currently \textit{Acremonium chrysogenum}).\textsuperscript{3,13} \(\beta\)-lactam drugs, including penicillins, cephalosporins, carbapenems, and monobactams, were named \(\beta\)-lactams according to their structure, \(\beta\)-lactam ring, a four-membered cyclic amide.\textsuperscript{3}
1.3.1 Penicillins

Each penicillin molecule contains β-lactam ring attached to a thiazolidine ring (Figure 1-2). The acyl side chain (R) attached to the amino group determines the susceptibility of each penicillin to degradation or enzymatic inactivation by bacterial β-lactamases and antibacterial activity of molecule.\(^{12-13}\) Hydrolysis is the main cause of penicillin degradation. Some penicillins are rapidly hydrolyzed by gastric acid, thus making them unsuitable for oral administration.\(^{13}\)

Penicillins are distinguished on the basis of structure and antibacterial activity into four groups:

(1) Natural penicillins, such as penicillin, are made from mold cultures. They are mainly active against Gram positive and some Gram negative bacteria including *Streptococci* spp., *Staphylococci* spp., *Actinomyces* spp., *Bacillus* spp., *Clostridium* spp., *Fusobacterium* spp., *Listeria monocytogenes*, *Pasteurella multocida* and *Haemophilus*
influenza but not *Pseudomonas* spp., most Enterobacteriaceae, or penicillinase-producing *Staphylococcus* spp.

(2) Aminopenicillins, such as ampicillin and amoxicillin, are semisynthetic derivatives of natural penicillins. They retain activity of natural penicillins but have increased activity against some Enterobacteriaceae including *E. coli*, *Proteus mirabilis*, and *Salmonella* spp. They are inactive against *Pseudomonas* spp., *Bacteroides fragilis* and penicillinase-producing *Staphylococcus* spp.

(3) Penicillinase-resistant penicillins, such as oxacillin, cloxacillin, have a ring structure attached to carbonyl carbon of the amide side chain. The substituents on the ring protect lactam ring from β-lactamases. This group of drugs has activity similar to, but slightly less than natural penicillins, but is active against penicillinase-producing *Staphylococcus* spp.

(4) Extended (broad) spectrum penicillins are further subgrouped into ureidopenicillins, such as piperacillin and azlocillin, and carboxypenicillins, such as ticarcillin and carbenicillin. This group of drugs has either a carboxylic acid group or a basic group at the α positive at R, providing them a broader spectrum of activity than other groups of penicillins. They are the most effective against Gram negative aerobic and anaerobic bacteria of all penicillin groups. They are active against many Enterobacteriaceae, *Pasteurella* spp., and some strains of *Pseudomonas* spp. Although these drugs are effective against Gram positive aerobic and anaerobic bacteria, their activity is generally less than that of natural penicillins and aminopenicillins.3, 12-13
1.3.2 Cephalosporins

Cephalosporin contains a 7-aminopenicillosporanic acid nucleus (or cephem nucleus) consisted of a four-membered \( \beta \)-lactam ring fused with a 6-membered dihydrothiazine ring (Figure 1-3). Cephalosporin nucleus is more resistant to \( \beta \)-lactamases than is the penicillin nucleus. Additions of various groups at R positions form derivatives with differences in antimicrobial activity, stability against \( \beta \)-lactamases, protein binding, intestinal absorption, metabolism and toxicity. Changes at position 7 (R2) alter \( \beta \)-lactamase activity and antibacterial properties, while changes in position 3 (R1) tend to alter metabolic stability and pharmacokinetic properties.\(^{13-14}\)

Cephalosporins are classified into 4 generations mainly based on the chronological development of the drugs. However, some generations are based on antimicrobial activity including \( \beta \)-lactamase stability and pharmacological properties.

1. First generation cephalosporins, such as cephalothin, cephalexin and cefazolin, have the similar spectrum of activity to that of aminopenicillins against \textit{Streptococci} spp., \textit{E. coli}, \textit{Proteus mirabilis}, and \textit{Klebsiella} spp., but have better activity against \textit{Staphylococci} spp. However, they are not generally active against anaerobic bacteria, \textit{Pseudomonas} spp., \textit{Enterococcus} spp., \textit{Enterobacter} spp., other \textit{Proteus} and \textit{Serratia} spp.

2. Second generation cephalosporins, such as cefoxitin and cefotetan tend to be active against the same bacteria as the first generation cephalosporins. However, the activity against Gram negative is enhanced. Second generation cephalosporins are considered in 3 groups; true cephalosporins, cephamycins, and carbacephems.
Cephamycins are chemically related to cephalosporins, differing primarily in possessing a 7 α-methoxy group (R3) which enhances their stability to certain β-lactamases and enhances activity against Gram negative bacteria and rods. Cephamycins are obtained from *Streptomyces* spp., which are bacteria more than fungi. These second generation cephalosporins are active against some strains of *Enterobacter* spp., *E. coli*, *Klebsiella* spp., *Proteus* and *Serratia* that are resistant to first generation cephalosporins.

(3) Third generation cephalosporins, such as cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, typically have less activity against Staphylococci but have greater activity against Gram negative bacteria. Most third generation cephalosporins are active against *Pseudomonas* spp. Third generation cephalosporins increase resistance to β-lactamase inactivation and have better penetrability through porins, particularly of Gram negative bacteria.

(4) Fourth generation cephalosporins, such as cefepime, are characterized by a quaternary ammonium substituent at C-3’ of cephem nucleus. They have higher potencies against the members of Enterobacteriaceae compared to other cephalosporins. In addition, they are active against Gram positive cocci, broad array of Gram negative, including *P. aeruginosa*, many Enterobacteriaceae, and methicillin-susceptible *S. aureus*, *S. pneumoniae* and other *Streptococci*. However, they are not active against methicillin-resistant strains. Fourth generation cephalosporins have increased stability to class 1b β-lactamases. Moreover, they possess enhanced penetration through porins in outer membrane of Gram negative bacteria, compared to thos of third generation
cephalosporins, due to the presence of positive charged quaternary ammonium ion at position 3 of cephem nucleus.\textsuperscript{3, 13-14}

### 1.3.3 Mechanism of action

Beta-lactam antibacterial drugs exert their bactericidal effects by preventing of bacterial cell wall synthesis and disrupting bacterial cell wall integrity, at the final step of peptidoglycan synthesis; the transpeptidation reaction that cross-links the peptide side chains of polysaccharide peptidoglycan backbone. β-lactam ring is a structural analog to the final peptide bridge (D-alanine-D-alanine) that cross-links the peptidoglycan chains that compose the bacterial cell wall. Thus they inhibit the active site of transpeptidase and inhibit other inner membrane proteins that may have a role in peptidoglycan synthesis. These transpeptidase and these other proteins are called penicillin-binding proteins (PBPs). PBPs, in particular transglycosylase, catalyze cross-linkage of glycopeptides polymer units that form cell wall. The final result of β-lactam binding to PBPs and inhibiting cross-linking is to trigger endogenous enzymes, such as autolysin, that degrade peptidoglycan. Normally peptidoglycan cell wall prevents bacteria from bursting in response to high osmotic strength of cytoplasmic contents relative to external medium leading to bacterial lysis. However, β-lactams cause lysis only to cells which are undergoing active cell wall synthesis because these cells are in the active process of transpeptidation.\textsuperscript{5, 10-13, 15}
Spectrum of activity of β-lactam drugs to Gram positive and Gram negative bacteria differ in various β-lactam drugs, in part, due to the different affinity of β-lactam drugs for the different PBPs, differences in receptor sites (PBPs), the relative amount of peptidoglycan present, the ability of drugs to penetrate outer cell membrane of Gram negative, and resistance to different types of β-lactamase enzymes.\textsuperscript{12, 13}

1.4 Fluoroquinolones

Fluoroquinolones (FQ), also known as quinolones, 4-quinolones, pyridine-β-carboxylic acid and quinolone carboxylic acid, are a large group of synthetic antimicrobial agents.\textsuperscript{16, 17} Quinolones were first discovered as a by-product formed during the synthesis of an antimalarial agent, chloroquine, as 7-chloro-1-ethyl-1,4 dihydro-4-oxo-3-quinoline carboxylic acid. Subsequently, 7-methyl-1-ethyl-1,4 dihydro-4-oxo-3-quinoline carboxylic acid, which was further called nalidixic acid, was discovered by Lesher et al in 1962.\textsuperscript{3, 18} The chemical structure of quinolones is based on the 1,4dihydro-4-oxo-pyridine molecule, a carboxylic acid substituent at position 3 and carbonyl group at position 4, which is essential for antibacterial activity of quinolones.\textsuperscript{3, 19-20}

Quinolones are classified on the basis of chemical structure or biological activity. Based on chemical structure, they are divided into 4 groups; (1) Group I composed of monocyclic derivatives, (2) Group II, which consists of the majority of FQ, is composed of bicyclic derivatives. There are 2 subgroups according to substitutions at position 8 of quinolone nucleus (3) Group III, which includes marbofloxacin, composed of tricyclic
derivatives and (4) Group IV, which a few has been synthesized, is comprised of quadricyclic derivatives.\textsuperscript{16}

Based on biological activity, quinolones are classified into 3 generations;

(1) First generation quinolones, such as nalidixic acid, are generally active against Gram negative bacteria such as \textit{Enterobacteriacea} spp., but not \textit{Pseudomonas} spp. These quinolones reach the high concentration in urinary tract, thus they have been used to treat urinary tract infections. However, first generation quinolones lack activity against Gram positive cocci and strict anaerobes. However, their poor absorption following oral administration, their propensity to bind to various proteins, and poor tolerance limit their clinically use.\textsuperscript{16, 19} Flumequin, another first generation quinolone, was synthesized by substitution of a fluorine at position 6, making it active against Gram positive and also improved pharmacokinetic. However, it was not developed as a marketed drug.\textsuperscript{3, 16, 19}

(2) Second generation quinolones, a majority of FQ such as Norfloxacin, ciprofloxacin, ofloxacin and enrofloxacin, were developed by incorporating a cyclic diamine, piperazinyl or methylpiperazinyl moiety, at position 7 and a fluorine atom at position 6 in quinolone nucleus in addition to a carboxy group at C-3 and a keto group at C-4.\textsuperscript{3, 19} This group of drugs are called FQ due to the substitution of a fluorine at position 6 (Figure 1-3).\textsuperscript{10, 16} Substituting a piperazinyl ring for the methyl group at position 7 provides better activity against aerobic Gram negative bacteria and increases activity against Staphylococci and pseudomonads.\textsuperscript{3, 16} Second generation quinolones are well absorbed from gastrointestinal tract and have increased tissue distribution, providing adequate levels in blood to promote their use for systemic infections.\textsuperscript{3, 18, 21} They also
have great activity against Gram negative bacteria such as *Enterobacteriaceae* and *Pseudomonas* spp., and moderate activity against Gram positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. However, they lack activity against anaerobes.19

(3) Third generation quinolones, including levofloxacin, moxifloxacin and pradofloxacin, are different from FQ by the substituents at positions 1, 7 and 8 of quinolone nucleus. Addition of a methoxy group at C-8 confers targeting of DNA gyrase and topoisomerase IV, thereby reducing emergence of resistant organisms.3 This group of drugs has significantly improved activity against Gram positive cocci and strict anaerobes, such as *Streptococcus* spp., especially *Streptococcus pneumoniae*.3, 16, 19

![Chemical structures of Enrofloxacin and Ciprofloxacin](image)

Figure 1-3 Representatives of second generation quinolones (fluoroquinolones; FQ) with fluorine substitution at position 6.
1.4.1 Mechanism of action

In most bacterial cells, chromosomes exist as circular double-stranded DNA. This circular DNA is compacted into supercoiled domains that must be relaxed and strands separated for transcription and translation.\(^3,\ 20\) Topoisomerase enzymes catalyze changes in supercoiling of the DNA. Topoisomerase I catalyzes single-strand nick to relieve or introduce supercoiling by 1 turn whereas topoisomerase II, including DNA gyrase, catalyzes double-stranded break. Thus, topoisomerase II alters supercoiling by two turns.\(^16\)

DNA gyrase is the only bacterial enzyme that introduces negative superhelical twists (negative supercoil) into covalently closed circular DNA.\(^22\) Negative supercoiled DNA is important for initiation and elongation of DNA replication by removing positive supercoiling ahead of the replication fork.\(^3,\ 22-23\) DNA gyrase is composed of two A and two B subunits encoded by \(gyrA\) and \(gyrB\) genes (GyrA2B2).\(^16,\ 20-21,\ 23\) Gyrase binds to DNA and catalyzes double-stranded breaks and passes the unbroken double-stranded DNA through the opening to alter the supercoiling. Activity of DNA gyrase is dependent on ATP hydrolysis which serves to reset the enzyme for the next round of catalysis.\(^16,\ 23\) DNA gyrase plays at least 4 roles in chromosome functions; (1) to maintain a negative supercoiling level, which activates the chromosome for all process involving strand separation, (2) to facilitate the movement of replication fork and transcription complexes through DNA by adding negative supercoils ahead of the replication fork, (3) to remove knots from DNA, (4) to help bend and fold DNA.\(^23\)
Topoisomerase IV, a homolog of DNA gyrase, is composed of two parC and two parE subunits encoded for \textit{parC} and \textit{parE} genes respectively (ParC2E2). GyrA is homologous to ParC, and GyrB is homologous to ParE.\textsuperscript{16,20,24} Topoisomerase IV acts at the terminal stages of DNA replication to separate the mother and daughter chromosomes following DNA replication.\textsuperscript{16,22-23} Both DNA gyrase and topoisomerase IV cleave double-stranded DNA in both strands and then transport another segment of double stranded DNA through the cleaved DNA segment before re-ligating the DNA. The difference is that DNA gyrase removes the positive supercoils in front of the replication fork, while topoisomerase IV decatenates behind the replication fork.\textsuperscript{3}

Quinolones are the only direct inhibitors of DNA synthesis. They inhibit DNA replication by interacting with 2 related but functionally distinct type II topoisomerase enzymes, DNA gyrase and topoisomerase IV. They bind to the enzyme-DNA complex, stabilizing DNA strand breaks created by DNA gyrase and topoisomerase IV.\textsuperscript{20,25} DNA gyrase is a primary target in Gram negative bacteria of the first and second generation quinolones, whereas topoisomerase IV is a primary target of the third generation quinolones in Gram positive bacteria. This suggests that chemical structure of quinolones determines the mode of antibacterial action.\textsuperscript{3}

Mammals are generally resistant to the killing effect of quinolone antimicrobials since topoisomerase II in mammalian cells is not inhibited until the drug concentration reaches 100-1000 µg/ml, while bacteria are inhibited by concentrations less than 0.1-10 µg/ml.\textsuperscript{20}
1.5 Antimicrobial resistance

Antimicrobial resistance is a relative term. There are various definitions of antimicrobial resistance, which are based on different criteria.\textsuperscript{1,3} The most commonly used definitions are based on either microbiological (\textit{in vitro} resistance) or clinical (\textit{in vivo} resistance) criteria. According to microbiological definition, a strain is defined as resistant when it grows in the presence of higher concentrations of drug compared with phylogenetically related strain. For a clinical definition, a strain is defined as resistant when it survives antimicrobial therapy. However, a strain can be either resistant or sensitive to treatment depending on its location, dosage, mode of drug administration, tissue distribution of the drug and state of immune system under treatment.\textsuperscript{1}

1.5.1 Acquisition of Antimicrobial Resistance

Antimicrobial resistance can be either intrinsic or acquired. Intrinsic resistance is a natural phenomenon which occurs in the absence of selection pressure.\textsuperscript{26} This allows tolerance of a particular drug or antimicrobial class by all members of a bacterial group. Indeed, this refers to the insensitivity since it occurs in bacteria that have never been susceptible to the drug, for example, \textit{Pseudomonas aeruginosa} has low membrane permeability that leads to its innate resistance to many antibiotics.\textsuperscript{1,10} Acquired resistance is a major threat since it causes emergence and spread of resistance in initially susceptible bacterial population and consequently cause them to be less susceptible or not at all susceptible to these antimicrobial drugs. This is associated with only some strains of a
particular bacterial genus or species. Acquisition is due to a genetic change in bacterial genome, which can be a consequence of mutations of normal cellular genes (or endogenous resistance) and selection, or by acquiring the genetic information that encodes resistance from other bacteria (or exogenous resistance). Resistance can also result from a combination of mutational and gene transfer events.\textsuperscript{1, 3, 10, 26-28}

Acquired bacterial antimicrobial resistance from mutation may cause resistance by (1) altering the target protein structure to which the antibacterial agent binds by modifying or eliminating the binding site, (2) upregulating the production of enzymes that inactivate the antimicrobial agent, (3) downregulating or altering an outer membrane protein channel that the drug requires for cell entry, and (4) upregulating pumps that expel the drug from the cell. Strains of bacteria carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical transfer of resistance genes.\textsuperscript{27-28}

The acquisition of mobile DNA elements, in addition to mutations in the genes, has accelerated the exchange of resistance in bacteria, which is termed horizontal transfer of resistance genes. This may occur between strains of the same species or between different bacterial species or genera. Three mechanisms of genetic exchange, conjugation, transduction, and transformation, are identified as horizontal transfer of resistance genes.\textsuperscript{27-28}

Conjugation is the process of self-transfer of genetic material; mobilizable DNA molecules such as plasmids and conjugative transposons, from a donor to a recipient
through direct cell-to-cell contact. Close contact between donor and recipient is the major requirement for efficient conjugation. The self-transmissable conjugative F-plasmid of *E. coli* is the best-known example of an autonomously replicating molecule, which encodes all necessary factors required for conjugation. Conjugation can also mediate chromosomal exchange following the integration of a self-transmissible plasmid into the bacterial chromosome.5, 28-30

Transduction, on the other hand, is the process by which DNA is transferred from one bacterium to another by a virus (bacteriophage) that infects bacteria. When bacteriophages infect a bacterial cell by injection of their DNA, the phage DNA can direct the production of the new phage particles in the new host cell, including expression of phage-borne genes which leads to replication of the phage DNA and production of new phage particles. These new phage progenies are then released from the bacterial cell (lytic cycle). In contrast, phage DNA may integrate into the chromosomal DNA of the host cell as prophage and remain there as an inactive state for long period (lysogenic cycle). External factors such as UV-irradiation can activate the prophage and initiate a lytic cycle.28-29

Transformation, the first mechanism of DNA transfer discovered among prokaryotes, involves scavenging of naked DNA by a bacterium after the death and deterioration of a nearby bacterium. The free DNA in a dead bacterium degrades and is broken into fragments released into the surrounding milieu, which can be taken up by the competent recipient cells. If antimicrobial resistance genes are included in the degraded DNA, they can be taken up by a nearby bacterium and incorporated into the bacterial
genome. However, this mechanism is considered to play only a limited role in the transfer of resistance genes \textit{in vivo}.\textsuperscript{28-29}

Generally, transformation and transduction do not require viability of the donor cells, nor linkage in time and space between donor and recipient as in conjugation. However, both transformation and transduction require homology between donor and recipient DNA for the recombination, while conjugation can occur when the donor and recipient are from different species. In addition, transduction requires high host (receptor) specificity for bacteriophages and therefore is usually restricted to a closely related group of bacteria.\textsuperscript{1, 5}

\subsection*{1.5.2 Mechanisms of Antimicrobial Resistance}

Bacteria have developed various mechanisms to neutralize the action of antimicrobial drugs and acquisition of resistance (Figure 1-4).\textsuperscript{1, 3} There are three main mechanisms in which bacteria develop to resist the antimicrobial therapy; (1) prevention of accumulation of antimicrobial drugs either by decreasing uptake or increasing efflux, (2) enzymatic inactivation of antimicrobial drugs by modification of their structure, and (3) qualitative alteration of the target that reduces the affinity for antibiotics either by mutation or by target modification, or by overproduction of target.\textsuperscript{3, 10}
(1) Prevention of accumulation of antimicrobial drugs either by decreasing uptake or increasing efflux:

Efflux pumps are transmembrane proteins that mediate active efflux.\textsuperscript{1} They are naturally present in both susceptible and resistant bacteria.\textsuperscript{3,28} Active efflux is an important mechanism of resistance to TC, FQ and macrolides. The antimicrobial drugs that inhibit protein synthesis must enter cell cytoplasm and accumulate to a sufficient concentration to allow them to bind ribosome. Active efflux system, an energy-dependent system, pumps drugs out of cytoplasm as rapid as it is taken up, resulting in decreased accumulation of drug in the cell.\textsuperscript{3,5} In Gram negative bacilli, efflux pumps are often encoded chromosomally and most strains carry genetic determinants for several pumps, establishing a level of intrinsic resistance to a multitude of antimicrobials.\textsuperscript{28} Some efflux systems have a narrow range of structurally related substrates, such as TetB tetracycline transporter in \textit{E. coli} which is specific to tetracycline and a narrow range of close structural analogs.\textsuperscript{3,28} However, many efflux systems have a broad range of structurally unrelated antimicrobial agents, leading to multidrug resistance (MDR) which will be discussed later in this chapter.

In gram negative bacteria, hydrophilic drugs enter bacterial cells through the porins on outer membrane, while hydrophobic drugs diffuse through phospholipid layer.\textsuperscript{1} Outer membrane porins (Omps) provide channels for molecules to enter the cell membrane in Gram negative bacteria, with different permeability depending on charge, shape, and size of the molecules.\textsuperscript{5,28} This is an example why vancomycin, an effective drug for Gram positive bacteria, that has the bulky molecule is not effective in Gram negative bacteria. Mutation in porins or porin loss can further limit the diffusion of
antimicrobial drugs through Gram negative bacteria. Although mutation in porins increases only 5-10 fold in resistance compared to 50-100 fold from other mechanisms, it can cause a clinical disaster since the highest concentrations of antimicrobial achievable at site of infection is sometimes less than 5 times the level required to kill or inhibit bacterial growth.5,26

Figure 1-4 Schematic representative of the mechanisms of antimicrobial resistance

(2) Enzymatic inactivation of antimicrobial drugs by modification of their structure:

Some bacteria have ability to possess enzymes to inactivate antimicrobials. Enzyme inactivation is an important mechanism of resistance only to natural antibiotics such as β-lactams, Aminoglycosides and chloramphenicol (no enzyme that can hydrolyze
or modify synthetic antimicrobial drugs such as sulfonamides, trimethoprim, or FQ have been reported.\textsuperscript{1, 3, 10} These enzymes modify active nucleus of the drug, making it unable to bind its target and lose antibacterial activity. Modification may consist of either cleavage of the molecule or addition of a chemical group.\textsuperscript{1}

For β-lactam antimicrobials, β-lactamases are a major mechanism of resistance. They catalyze the opening of β-lactam nucleus by cleaving C-N bond of β-lactam ring (Figure 1-5), resulting in an inactive product that cannot bind to PBPs and impede cross-linking of peptidoglycan during cell wall synthesis.\textsuperscript{1, 3, 5, 30} β-lactamase genes are located in chromosome, plasmids or transposons. They are secreted into periplasmic space by Gram negative bacteria, and into extracellular millieu by Gram positive bacteria. Since gram negative bacteria confine their β-lactamases into periplasm and have porins that restrict entry to the cell, thus they can achieve the same level of resistance with a lower level of enzyme than Gram positive. Unlike porin mutations that confer resistance to many different drugs, β-lactamases are much more specific and are usually active only against β-lactam derivatives.\textsuperscript{5, 29}

Enzymatic inactivation is also a major mechanism of aminoglycoside resistance. It involves aminoglycoside-modifying enzymes which inactivate the drugs by adding groups such as phosphoryl, adenyl, or acetyl group. There are 3 types of aminoglycoside-modifying enzymes; \textit{N}-acetyltransferases which transfer acetyl group from acetyl coenzyme A; aminoglycoside \textit{O}-phosphoryltransferases which transfer phosphoryl group from ATP, and aminoglycoside \textit{O}-nucleotidyltransferases which transfer a nucleotide from nucleotide triphosphate, resulting in a chemically modified drug that binds poorly to
ribosomes and is less rapidly taken up by the cell.\textsuperscript{1, 3, 27} Aminoglycoside-modifying enzymes are located on the outside of the cytoplasmic membrane in Gram negative bacteria.\textsuperscript{5}

For chloramphenicol, chloramphenicol acetyltransferases enzymes inactivate chloramphenicol by transferring acetyl group from acetyl coenzyme A. There are several \textit{cat} genes encoding different chloramphenicol acetyltransferases.\textsuperscript{3, 5}

![β-lactamase mechanism](image.png)

Figure 1-5 The mechanism of action of β-lactamase enzymes

(3) Modification or protection of antimicrobial targets:

Antimicrobial drug target can be structurally modified or replaced so that the drug can no longer bind and exert its activity on the cell. The examples of this are the mechanisms of resistance to β-lactams and FQ.

In β-lactam resistance, particularly in Gram positive bacteria, alteration of drug target is a second mechanism of resistance, in which binding specificity of PBPs is altered or mutated so they can no longer bind the drugs. The best characterized gene is \textit{mecA} gene in \textit{Staphylococcus aureus}.\textsuperscript{5, 27}

Resistance to FQ usually involves point mutations that alter binding targets of drugs, resulting in lowered binding affinity. Multiple point mutations can lead to the high
levels of resistance, as in quinolone resistance-determining region (QRDR) of DNA topoisomerase genes, such as \textit{gyrA}, \textit{gyrB}, and \textit{parC} where multiple mutations cause higher MIC and therefore decreased susceptibility to FQ\textsuperscript{5,27}.

### 1.5.3 Resistance to \(\beta\)-lactam drugs

Resistance to \(\beta\)-lactam antimicrobial drugs is most frequently mediated by the presence of \(\beta\)-lactamase enzymes, following by mutations in PBP\textsubscript{s}\textsuperscript{29,32-33}. Resistance due to reduced uptake from alterations in the outer membrane of gram negative bacteria or active efflux, however, is less frequently reported\textsuperscript{29,32}.

(1) Enzymatic inactivation:

Presence of \(\beta\)-lactamase enzymes is the main mechanism that confers resistance to \(\beta\)-lactam drugs. There are several systems to classify \(\beta\)-lactamas\textsuperscript{es\textsuperscript{29,30,32,34}}. One of the classification schemes is based on their nucleotide sequence, A to D (Figure 1-2). Enzymes in classes A, C and D have a serine at their active site, whereas enzymes in class B have four zinc atoms at their active site. In general, class A enzymes are highly active against benzylpenicillins. Extended-spectrum \(\beta\)-lactamase enzymes (ESBL\textsubscript{s}) are also included in this group. ESBL\textsubscript{s} not only inactivate benzylpenicillins, but also cephalosporins and/or monobactams. Class B enzymes are active against both penicillins and cephalosporins. Some of enzymes in this group are also active against carbapenems. Genes for class C enzymes are inducible, but mutations in these genes lead to
overexpression of the enzymes. Class D \(\beta\)-lactamase enzymes are composed of the OXA-type enzymes, which are capable of hydrolyzing oxacillin and its derivatives. Genes encoding \(\beta\)-lactamase enzymes are located either on plasmids or bacterial chromosome. They are also found in both Gram positive and Gram negative bacteria.\textsuperscript{32, 34}

Another classification scheme, proposed by Bush, Jacoby and Medeiros in 1995, classifies \(\beta\)-lactamase enzymes into four classes on the basis of the substrate spectrum and inhibition by clavulanic acid (1-4) (Table 1-2).\textsuperscript{29, 30, 33-34} Only \(\beta\)-lactamase enzymes of class 2a are present in Gram positive bacteria, as all other \(\beta\)-lactamasases are mainly found in Gram negative bacteria.\textsuperscript{29, 30, 33-34} \(\beta\)-lactamase enzymes of Gram negative bacteria are secreted into periplasmic space, while \(\beta\)-lactamases of Gram positive bacteria are secreted from the cell.\textsuperscript{5, 29} Most \(\beta\)-lactamase enzymes of Gram negative bacteria, except class 1 enzymes, are constitutively expressed whereas class 2b \(\beta\)-lactamases of Gram positive bacteria are usually inducible.\textsuperscript{29}

2) Modification of \(\beta\)-lactam targets:

Resistance to \(\beta\)-lactam antimicrobial drugs can also develop through the production of modified PBPs, particularly in Gram positive bacteria. In \textit{Staphylococcus} spp., resistance to methicillin occurs by acquisition of \textit{mecA} gene, a chromosomal gene which encodes a new high-molecular weight PBP called PBP2A. This new PBP2A has low affinity for \(\beta\)-lactam antimicrobial drugs especially methicillin, leading to methicillin resistant \textit{Staphylococcus aureus} (MRSA).\textsuperscript{3-4, 29} MRSA is inducible and MRSA isolates are resistant not only to penicillins, but also to other \(\beta\)-lactam antimicrobials including cephalosporins, monobactams and carbapenems.\textsuperscript{3, 29}
In *Streptococcus pneumoniae*, resistance to penicillins is mediated by target alteration, not by \(\beta\)-lactamase enzymes.\(^{29, 35}\) Penicillin resistant *S. pneumoniae* occurs by acquisition of mosaic genes encoding modified PBPs, such as PBP2X, PBP2B, PBP1A, by transformation. The extensive modification of multiple PBPs cause high level resistance to penicillins.\(^{35}\)

Table 1-2 Classification schemes of \(\beta\)-lactamase enzymes

<table>
<thead>
<tr>
<th>Classifications</th>
<th>Bush-Jacoby-Medeiros</th>
<th>Molecular class</th>
<th>(\beta)-lactam drugs (substrates)</th>
<th>Representative enzymes</th>
<th>Inhibited by clavulanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>Cephalosporins</td>
<td>AmpC from Gram negative bacteria</td>
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<tr>
<td>2a</td>
<td>A</td>
<td>Penicillins</td>
<td>Penicillinases from Gram positive bacteria</td>
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<tr>
<td>2b</td>
<td>A</td>
<td>Penicillins, cephalosporins,</td>
<td>TEM-1, TEM-2, SHV-1</td>
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<td>+</td>
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<tr>
<td>2be</td>
<td>A</td>
<td>Penicillins, narrow-spectrum and extended-spectrum cephalosporins, monobactams</td>
<td>TEM-3 to TEM-27, SHV-2 to SHV-7, K1</td>
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<td>+</td>
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<tr>
<td>2br</td>
<td>A</td>
<td>Penicillins</td>
<td>TEM-30 to TEM-36, TRC-1</td>
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<td>±</td>
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<td>Penicillins,</td>
<td>PSE-1, PSE-3, PSE-4, BRO-1, BRO-2</td>
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<td>+</td>
</tr>
<tr>
<td>2d</td>
<td>D</td>
<td>Penicillins, cephalosporins, oxacillin</td>
<td>OXA-1 to OXA-11, PSE-2 (OXA-10)</td>
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<td>±</td>
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<td>2e</td>
<td>A</td>
<td>Cephalosporins</td>
<td>Inducible cephalosporinases from <em>Proteus vulgaris</em></td>
<td></td>
<td>+</td>
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<tr>
<td>2f</td>
<td>A</td>
<td>Penicillins, cephalosporins, monobactams, carbapenems</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>Penicillins, cephalosporins, monobactams, carbapenems</td>
<td>L1, CerA, IMP-1</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>Penicillins</td>
<td>Penicillinase from <em>Pseudomonas cepacia</em></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
(3) Reduced uptake of β-lactam drugs

Resistance to β-lactam antimicrobial drugs by their reduced uptake are mediated by either decreased expression or the structural alteration of porins, such as OmpF and OmpC, by which β-lactam drugs pass through the outer membrane in Gram negative bacteria. In Pseudomonas aeruginosa, resistance to Imipenem is based on the loss of porin OprD.29

(4) Active efflux:

Resistance to β-lactam drugs can also be mediated by active flux such as AcrAB/TolC pumps in Salmonella and E. coli spp., and MexAB/OprM in Pseudomonas spp. However, most of efflux pumps that mediate β-lactam resistance have broad tolerance and confer multidrug resistance (MDR) phenotypes.3, 29 Details of MDR efflux pumps will be discussed later in this chapter.

1.5.4 Resistance to fluoroquinolones

Resistance to FQ is mainly based on mutations which render the target resistant to the drugs or decreased intracellular drug accumulation. However, other mechanisms including active efflux, decreased drug uptake, and modifications of targets (DNA gyrase protection) also contribute to quinolone resistance, while enzymatic inactivation in FQ resistance has not yet been reported.24, 29, 31, 39
(1) Modifications of target structures

Point mutations encoding single amino acid changes in either DNA gyrase or topoisomerase IV can cause quinolone resistance. Mutations of these enzymes are varied among Gram positive and Gram negative bacteria. The effects of different mutations on resistance differ with respect to the various FQ. DNA gyrase seems to be the primary target in Gram negative bacteria whereas topoisomerase IV seems to be main target in Gram positive bacteria. In addition, levels of quinolone resistance are dependent on whether alterations are on the primary target, the secondary target, or both. Mutations in the primary target precede those in the secondary target, in a stepwise selection for resistance; mutations in both targets produce higher levels of resistance.

The resistance mutations are commonly located in the amino terminal domain of GyrA and ParC and are in proximity to the active tyrosine. This domain has been termed Quinolone resistance determining region (QRDR) of GyrA and ParC. In E. coli, the most common sites of GyrA mutation is at serine (Ser) 83, that may be changed to tryptophan (Trp), leucine (Leu), alanine or other amino acids, and aspartate (Asp) 87, and at Ser 79 and Asp 83 for ParC. This mutation site is also at equivalent positions of GyrA of other species, or equivalent positions of ParC. Once one or two substitutions within QRDR of GyrA have accumulated, further substitutions in QRDR of ParC can lead to incremental increases in resistance to quinolones. Mutations in specific domains of GyrB and ParE also mediate FQ resistance, although they are substantially less common.
Another FQ resistance that involves target modification and protection of DNA gyrase is termed Qnr. Qnr proteins are plasmid-encoded, initially discovered by Martinez-Martinez and associates in 1998. Qnr proteins confer low level resistance to quinolone drugs. Thus far, 3 major Qnr proteins have been identified, QnrA, QnrB and QnrS with additional variants within each group that differ by a few amino acids. They are mostly found in Gram negative bacteria such as *Klebsiella* spp., *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Shigella* spp. and *Salmonella* spp. All Qnr proteins are members of a pentapeptide repeat family, in which either leucine or phenylalanine is present at almost every fifth amino acid and each pentapeptide repeat likely forms β sheet that is important for protein-protein interaction. Qnr proteins bind specifically to DNA gyrase, probably before the formation of cleavage complex (gyrase-DNA-quinolone complex) and protect it from the inhibitory activity of quinolones.

(2) Active efflux mediated FQ resistance

The expression or overexpression of energy-dependent efflux pumps can actively remove FQ from the cells. In general, efflux pump mediated FQ resistance mediates the low-level resistance to FQ. However, in combination of efflux pump and chromosomal mutations in GyrA, ParC genes, high level resistance to FQ has been reported. There are several efflux pumps that mediate FQ resistance in both Gram positive and Gram negative bacteria. Efflux pumps in Gram positive bacteria are multidrug transporters which are driven by the electrochemical proton gradient, such as
NorA in *S. aureus*. In *E. coli*, AcrAB/TolC, AcrD, YhiV and AcrSEF have been reported to mediate FQ resistance.

Recently, a plasmid-mediated efflux pump, QepA, has been reported in clinical *E. coli* isolates from Japan by Yamane and associates in 2007. QepA encodes an efflux pump belonging to the major facilitator subfamily (MSF). The MICs to quinolone drugs were 32 to 64-fold increased in *E. coli* experimental strains expressing QepA compared with the host strain, while MICs for other drugs such as ampicillin, erythromycin, kanamycin, tetracycline, and chemical substances such as acriflavine, carbonyl cyanide m-chlorophenylhydrazone, crystal violet, and sodium dodecyl sulfate were not affected. This indicates that QepA is a FQ-specific MSF-type efflux pump. Additive effect of QepA on FQ resistance mediated by mutations of DNA gyrase and/or topoisomerase IV may promote further spread of FQ resistant strains.

### 1.6 Multidrug Resistance (MDR)

Multidrug resistance (MDR) is the term which is used when an organism is resistant to 3 or more unrelated antibacterial drug classes. The main mechanism of MDR is active efflux which has the ability to extrude the structurally diverse compounds. The energy for the translocation process is derived from proton motive force or ATP hydrolysis.
1.6.1 Multidrug Resistance Efflux systems

MDR efflux systems can be either chromosomally or plasmid encoded. Generally, they have a rather broad substrate specificity and are able to handle antimicrobial agents of several different classes. MDR transporters are classified based on energy source into 2 divisions; (1) proton motive force-dependent secondary transporters, which function as the secondary transporters catalyzing drug-ion (H⁺ or Na⁺) antiport, an electrochemical gradient in which movement of proton ion drives the transport of substrate, and (2) ATP-binding transporters, which utilize ATP hydrolysis as the energy source to transport substrate out of the cell. However, MDR pumps can also be classified into 5 distinct families on the basis of the number of components that the pump has (either single or multiple), the number of transmembrane-spanning region that the transporter protein has, the energy source that the pump uses, and the type of substrate that the pump exports: (1) The major facilitator superfamily (MFS) which is responsible mostly for MDR in Gram positive bacteria and are not expressed in Gram negative bacteria under normal conditions; (2) Resistance-nodulation-cell division (RND) family, which are the main pumps in Gram negative bacteria; (3) Small multidrug resistance family (SMR), which is found in some of Gram positive bacteria and most are not expressed in Gram negative bacteria under normal conditions; (4) Multidrug and toxic compound extrusion family (MATE), which are the Na⁺/drug antiporters and found in Gram negative bacteria; (5) ATP-binding transporter superfamily, which is the only type of efflux pumps that utilizes ATP hydrolysis as the energy source.
An organism can express MDR efflux pumps from more than 1 family and 1 or more than 1 type of efflux pump belonging to the same family. Efflux is suspected to be the mechanism of MDR when there is a simultaneous increase in MICs of 3 or more antimicrobial drugs for a particular organism compared with MICs of them for the parent strain. Normally, increased MICs mediated by MDR efflux systems are typically 2-8-fold higher than MICs in the susceptible strains.51

1.6.2 MDR efflux systems in *Escherichia coli*

There are several classes of efflux pumps that can mediate MDR in *E. coli* (Table 1-3), however, the main efflux system belongs to pumps in RND family, AcrAB-TolC.47, 50-52 In general, efflux pumps in RND family can recognize and extrude positive-, negative- and neutral-charged molecules, hydrophobic organic solvents and lipids, and hydrophilic compounds like aminoglycoside antimicrobials.52

RND efflux pumps typically operate as part of a tripartite system (Figure 1-6) that is composed of (1) periplasmic membrane fusion protein (MFP), an accessory protein located in periplasmic space; (2) an outer membrane protein or outer membrane factor (OMF), an outer membrane channel located in the outer membrane of bacteria; and (3) a transporter (efflux) protein, located in the inner (cytoplasmic) membrane.47, 51

AcrAB-TolC tripartite in *E. coli* contains 12 transmembrane segments in a transmembrane domain (TMD) with a large periplasmic domain. It functions as a trimer with AcrB, AcrA, and TolC as a transporter, MFP, and OMF, respectively.47, 51-52
Monomers of AcrB have very limited contact with one another. AcrB, AcrA and TolC assemble into a mushroom-like structure that protrudes from the membrane into the periplasm. AcrAB-TolC systems utilize movement of H⁺ into cell to transport the substrate, including lipophilic β-lactams, FQ, nalidixic acid, chloramphenicol, tetracycline, rifampin, fusidic acid, novobiocin as well as sodium dodecyl sulfate, pine oil, acriflavine, acridine, bile salts, fatty acids, crystal violet, ethidium bromide, and organic solvents, out of the cell.50-51

Table 1-3 Summary of the efflux pumps that can mediate multidrug resistance in E. coli

<table>
<thead>
<tr>
<th>Family</th>
<th>Efflux pump</th>
<th>Substrates³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BLs</td>
</tr>
<tr>
<td>RND</td>
<td>AcrAB-TolC</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AcrEF-TolC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MdtABC</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>YhiUV</td>
<td>-</td>
</tr>
<tr>
<td>MFS</td>
<td>MdfA</td>
<td>-</td>
</tr>
<tr>
<td>SMR</td>
<td>EmrE</td>
<td>-</td>
</tr>
</tbody>
</table>

³BLs = β-lactams, FQ = fluoroquinolones, TET = tetracyclines, CML = chloramphenicol, AGs = aminoglycosides, TMP = trimethoprim, ERY = erythromycin, NOV = novobiocin, RIF = rifampin, PUR = puromycin
In *E. coli*, TolC can function as the port channel for different RND-family efflux pumps, and it can also interact with MFS transporters (such as EmrAB of *E. coli*) and ABC superfamily transporter (such as MacAB of *E. coli*). Among all Acr efflux pumps in *E. coli*, AcrAB-TolC has been found to be over expressed by clinical isolates. Overexpression of AcrAB-TolC alone does not confer clinical level of resistance, however, when a mutation(s) in a topoisomerase gene occurs in the same bacterium as increased efflux, such isolates are resistant to FQ such as ciprofloxacin.

Figure 1-6 Schematic representative of RND efflux pump of Gram negative bacteria.

(OM = outer membrane; PP = periplasmic space, CM = cytoplasmic membrane, OMP = outer membrane protein, MFP = membrane fusion protein)
1.7 Commensal flora

The normal flora, in general, refers to the microflora present at any site in a healthy human or animal. Normal Flora is present in the body shortly after birth, when the sterile fetus is colonized in the birth canal and by the immediate environment. Later, the microflora of the digestive tract is influenced by the food intake. Bacteria of the normal flora can be divided into 3 groups; (1) symbionts - which benefit both themselves and hosts, (2) commensal - which do not seem to benefit but not harm any hosts, and (3) opportunists - which may harm hosts and cause disease under certain circumstances.

1.7.1 Digestive tract and intestinal microflora

The digestive tract is usually populated by numerous bacteria, with different bacteria present in distinct part of the digestive tract. The oral cavity is one of the most favorable habitats for heterogenous microbes due to the presence of food particles and epithelial debris optimal for bacterial growth. The bacterial flora present here includes both strict anaerobes and facultative anaerobic bacteria. The stomach contains relatively low numbers of microorganisms, generally facultative anaerobes, due to its acidic environment.

In the intestine, the presence of normal flora is also distinct. The anterior part of small intestine (duodenum, jejunum) resembles the stomach in terms of its acidic and microbial content. Both stomach and anterior part of small intestine are practically
free of cultivable living microorganisms when they are empty, but harbor small numbers of organisms at least transiently with highest numbers shortly after eating. It mainly consists of Gram positive species. The distal part of small intestine (ileum) is less acidic and thus contains various and more complex microflora including substantial numbers of coliforms, Enterobacteriaceae, Bacteroides, Enterococcus and Lactobacillus spp.53, 55

The large intestine is densely populated by microorganisms. In the caecum aerobic species such as Enterococcus spp., and Lactobacillus spp and facultative anaerobic species like E. coli, comprise a significantly larger part of the total bacterial count.55

1.7.2 Digestive tract and intestinal microflora of dogs

The intestine of the dogs is colonized by a broad variety of microflora like other animals and humans. Presence of microflora in dog intestine is similar to that of human, originating from the mother and followed by microbes benefiting from breast-feeding and then drastically changing towards obligate anaerobes and greater diversity as foods are introduced.54

Dogs are carnivorous, thus the length of the intestine in relation to the body length is shorter and the motility is slower than in humans. However, the digestive tract of dog resembles that of humans in many ways. For example, both humans and dogs utilize intestinal microbiota in their physiology. In addition, the pH in the different compartments of the digestive tract of dogs is also comparable to those of humans: pH in the dog stomach is 3, in duodenum and jejunum 6, in ileum 7.5, in colon 6.5 and in
Moreover, the main bacteria commonly found in humans and dogs are the same, such as coliforms, *Clostridium* spp., *Bacteroides* spp., *Streptococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp., with the bacterial counts increasing towards the large intestine.  

### 1.7.3 Fecal coliforms and fecal *Escherichia coli*

Coliforms are gram-negative, facultative anaerobic, non-endospore forming rods that ferment lactose to produce acid and gas. Coliforms are one of the predominating bacterial groups in the humans and animals. *E. coli* is the most common and most studied. It is also considered an indicator of fecal contamination in food and water. Although *E. coli* may not cover more than a fraction (<1%) of the total count of the intestinal bacteria, it is significant as a pathogen for being one of the main causes of nosocomial infections, including urinary tract infections and diarrhea.

*E. coli* is a common inhabitant of the lower gastrointestinal tracts of mammals including humans and usually exists as a commensal. *E. coli* appears to develop resistance rapidly when exposed to selected antimicrobials and mechanisms of resistance often lead to MDR. In general, pathogenic *E. coli* has been classified regarding to virulence factors and enteric disease into 6 groups: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic or Verotoxigenic *E. coli* (EHEC or VTEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), Uropathogenic *E. coli* (UPEC) and diffusely adherent *E. coli* (DAEC). In dogs, a variety of *E. coli* strains including attaching and effacing *E. coli* (AEEC), necrotoxigenic
*E. coli* (NTEC), ETEC and VTEC have been associated with diarrheal diseases.\(^6^9\) Fecal *E. coli* from healthy dogs have been reported to possess virulence attributes regarded as markers of enteropathogenicity, such as *eae* and *sta* genes, heat-stable enterotoxin, verotoxin, and a hemolysin.\(^6^8,\ 7^0-7^2\) These findings suggest that potential pathogens are carried by healthy dogs.\(^7^1\) These indicate that *E. coli* is a good sentinel for the studies of antimicrobial resistance level of the fecal flora.\(^5^3\)

### 1.7.4 Role of commensal flora in antimicrobial resistance

Several factors such as stress from temperature, crowding, management, and antimicrobial therapy have been reported to contribute to the transfer of antimicrobial resistance in normal flora of bacteria.\(^5^3\) Among this, antimicrobial use is the most important factor for the emergence, selection and dissemination of antimicrobial resistance in both humans and animals.\(^7^3-7^5\) Antimicrobials exert selection pressure not only to pathogenic bacteria but also to commensal bacteria in the intestinal tract of humans and animals. This is partly because the intestine of animals represents an optimal environment for the selection and transfer of antimicrobial resistance genes.\(^7^6-7^7\) Fecal *E. coli* is considered to be a good indicator for selection pressure by antimicrobial therapy and for the transfer of antimicrobial resistance genes to pathogenic bacteria.\(^5^3,\ 5^5,\ 7^8\)

Various antimicrobial resistance genes such as ampicillin, tetracycline, sulfamethoxazole, trimethoprim, streptomycin and FQ resistance genes have been reported from commensal flora of intestinal tract of healthy animals. As a result, antimicrobial resistant commensal bacteria, including fecal *E. coli*, of healthy animals
constitute a reservoir of antimicrobial resistant genes that play an important role in resistance development in pathogenic bacteria.\textsuperscript{57, 78-82}

Resistant strains that are selected within the site of colonization under antibiotic pressure can be spread to other sites and to other individuals. Several studies have reported the evidence of transfer of bacteria, especially with antimicrobial resistance genes in \textit{E. coli}, between humans and animals.\textsuperscript{1, 75, 81-87} Further, it has been shown that resistant bacteria can persist in humans for a long time without any antibiotic pressure. In one study, high-level clarithromycin-resistant enterococci were isolated in patients up to four years after antibiotic treatment.\textsuperscript{88-89}

\textbf{1.7.5 Antimicrobial resistance in \textit{Escherichia coli} in companion animals}

The emergence of antimicrobial resistance in companion animals has been reported for more than forty years. In 1970s Hirsh identified MDR \textit{E. coli} isolates from urine of dogs and cats with cystitis. Later, several studies of antimicrobial resistance, including MDR, on companion animals mostly focused on pathogenic \textit{E. coli} from clinical \textit{E. coli} isolates, especially from urinary tract infection (UTI). Resistance to various antimicrobial drugs including β-lactams, the presence of ESBLs and FQ in companion animals has been reported in several countries. Prevalence of antimicrobial resistance from canine and feline clinical \textit{E. coli} isolates between 1989-1997 was reported in UK by Normand et al., and in about the same time as the studies of Lanz et al. in Switzerland and several studies in the US.\textsuperscript{83, 85, 90-95}
Methods of transfer of antimicrobial resistance such as plasmids and integrons in antimicrobial resistance including MDR were also reported in companion animals. Johnson et al. proposed that dog feces could serve as a reservoir of *E. coli* strains that cause extraintestinal infection in humans, due to the clonal commonality between fecal *E. coli* from dogs and clinical *E. coli* in humans.\textsuperscript{96-97} Close contact between humans and dogs increases the potential for transmission and exchange of resistant bacteria between humans and dogs, as well as the exchange or transfer of resistant genes to human pathogens.\textsuperscript{79} Transfer of resistance genes between animals and humans is another major factor of the worldwide spread of problematic antimicrobial resistance in both human and veterinary medicine.

### 1.7.6 Analysis of antimicrobial resistance

Antimicrobial resistance can be detected phenotypically and genotypically. Phenotypic methods for susceptibility testing, such as disk diffusion and tube dilution methods, usually provide necessary information for the clinical standpoint. However, they may be time-consuming and less suitable as a rapid diagnostic tool, as pure bacteria are required for the tests.\textsuperscript{98-102} The susceptibility of bacteria can be detected in both a quantitative and a qualitative way. Quantitative methods will result in data that can be related to actual concentrations of antimicrobials inhibiting the growth of bacteria such as MICs, while qualitative methods will categorize bacteria as susceptible, intermediate or resistant.\textsuperscript{101}
Detection of antimicrobial resistance genes requires a substantial level of specificity and sensitivity and in many cases speed can be requisite. Genotypic methods are faster and are useful to estimate the diversity, distribution, and underlying molecular pathways, as well as to locate the important reservoirs of antimicrobial resistance genes. Molecular methods also help to determine the location of the gene and to differentiate between horizontal gene transfer and clonal spread. Moreover, genotypic detection is not dependent upon phenotypic categories such as MICs for which breakpoints may vary between countries. Several molecular techniques used in detection of antimicrobial resistance genes and their expression are based on nucleic acid hybridization (southern blot) and amplification (polymerase chain reaction; PCR). Since its introduction in the mid 1980s by Kary Mullis, PCR is among the most frequently used methods to identify and characterize antimicrobial resistance genes due to its speed, accuracy and automation. Several molecular typing methods, such as plasmid profiling and ribotyping are very useful to detect the source and vehicle of transmission of resistance genes. Moreover, whole genome DNA fingerprints can be detected by various methods such as pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and arbitrary primed PCR multilocus fingerprinting methods.
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CHAPTER 2

IMPACT OF KENNEL ENVIRONMENT AND DOG SOURCE ON FECAL COLIFORMS ANTIMICROBIAL RESISTANCE

2.1 Introduction

Bacterial antimicrobial resistance has markedly curtailed successful antimicrobial therapy in humans or animals. The literature is replete with reports attempting to identify or describe multiple facets of the advent of resistance, using both spontaneous \(^1\text{-}^5\) and experimental \(^6\text{-}^8\) models. Species serving as experimental models include humans, dogs, cats, rats, pigs, chickens, and cows.\(^1,^2,^5\) Dogs may be a particularly appealing model if the information targets human or canine subjects. However, compared to laboratory rodents, husbandry and housing considerations are more complex when using a canine experimental model. For many studies, standard housing and husbandry protocols may be sufficient to assure minimal impact on study results. However, studies focusing on gastrointestinal microflora may not be as amenable to standard protocols.

The purpose of this report is to describe the impact of differences in dog source and animal husbandry on baseline fecal coliform characteristics among experimental groups of dogs. Among the examples for which dog source and husbandry are important are the studies which focus on the impact of antimicrobials on the intestinal
microbiota. Dogs were subjects of a study that described the impact of oral antimicrobial therapy on the advent of coliform fecal resistance. The study hypothesized that dog sources and animal husbandry impact the resistance patterns of fecal *E. coli* in healthy, antimicrobial-free dogs. This hypothesis was to be tested by comparison of baseline fecal coliform resistance to that after treatment with either amoxicillin or enrofloxacin at routine oral doses. The sources of dogs to be used were random, although animals had been housed in the same environment and were drug free for approximately 9 months.

Assessment of resistance in *E. coli* collected from fresh feces revealed an unacceptably high proportion to be resistant to amoxicillin, one of the test drugs. As such, these dogs could not be used to test the hypothesis. Two other populations of dogs were available for study, both purpose bred, but differing in husbandry techniques: one population was maintained in relatively restricted environment whereas the second was maintained in strictly controlled conditions. The purpose of this report is to describe the differences in fecal *E. coli* resistance under baseline conditions and to demonstrate the impact of differences on fecal *E. coli* populations.

### 2.2 Materials and Methods

#### 2.2.1 Animals and sample collections

Dogs to be studied were participants in studies unrelated to the present report. Investigators directing the studies allowed dogs to be sampled for this report. However, the condition of study precluded changes in the husbandry or any other conditions.
Accordingly, dogs could not be randomly assigned to husbandry groups based on source or any other condition. A total of twenty-four healthy adult hound dogs were studied, with 8 dogs representing each of three groups defined by source and husbandry techniques in which the dogs were maintained at the time of study:

Group 1 (G1) dogs were random source (class B) dogs, in which their original source and history prior to acquisition by Auburn University was unknown. Thus, history was limited to the period immediately following acquisition to the data collection, which was 9 months. During this 9 month period, no drugs were received by the dogs other than heartworm preventative medicine. Therefore, no dogs had received any antimicrobial therapy for at least 9 months. G1 animals were housed in a kennel that was climate controlled. However, the back half of each cage was open to the environment by extending from the housed area into an uncovered area. The entirety of each cage floor was cement. However, exposure to birds, rodents, insects or other vectors was possible. Dog-to-dog contact occurred between chain-link fences that separated each cage in the kennel and was permitted during cleaning when dogs were released from cages and intermingled in the central kennel area. Two of the 8 dogs did not move into the kennel until 2 weeks after baseline data collection was initiated in the remaining 6 animals, but they were studied as long as the others. Each cage was washed daily with water and the kennel was disinfected once every 2 weeks and pressure washed once every month. However, cleaning procedures were not specifically designed to avoid potential contamination of adjacent runs with debris (including food and feces) from surrounding cages.
Group 2 (G2) dogs were purpose-bred (class A) dogs, which were raised in a closed colony and had a known history of no drug use other than that stated for G1. G2 dogs were housed in limited access kennel. Cages were as described for G1, but access to the kennel was limited to study and facility personnel only. Cleansing procedures were as with G1.

Group 3 (G3) dogs were purpose-bred (class A) dogs similar to G2. However, their housing differed from G1 and G2 in that the kennel is a climate controlled, indoor kennel with access to the kennel that was limited to immediate study personnel \( (n \leq 2) \). Animals were never exposed to the environment or other dogs and cleaning procedures were designed to assure lack of exposure to debris from adjacent kennels. The summary of different dog sources, kennel environments and husbandry are in Table 2-1.

### 2.2.2 Total and resistant coliform counts

Fecal samples were collected once a week for 4 to 6 weeks (for 2 G1 dogs; the timing of sample collection was offset two weeks). Fresh fecal samples were collected from the inner part of feces from the cage floor (approximately 1 hr after feeding) into sterile containers. The samples were collected at the same time each day and processed within 2 hours after collection.
Table 2-1 Summary of different dog sources, kennel environments and husbandry in each group of dogs in this study

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source</strong></td>
<td>Random (Class B)</td>
<td>Purpose bred (class A)</td>
<td>Purpose bred (class A)</td>
</tr>
<tr>
<td><strong>Dog to dog contact</strong></td>
<td>Between cages</td>
<td>Between cages</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>During cleaning</td>
<td>During cleaning</td>
<td></td>
</tr>
<tr>
<td></td>
<td>During play time</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exposure to environment</strong></td>
<td>Through the back half of the cage</td>
<td>Through the back half of the cage</td>
<td>None</td>
</tr>
<tr>
<td><strong>Cleansing procedure</strong></td>
<td>Daily washing of runs with water.</td>
<td>Daily washing of runs with water.</td>
<td>Daily washing of runs with water.</td>
</tr>
<tr>
<td></td>
<td>Sanitizing every 2 weeks.</td>
<td>Sanitizing every 2 weeks.</td>
<td>Sanitizing every week.</td>
</tr>
<tr>
<td></td>
<td>Pressure washing once a month.</td>
<td>Pressure washing once a month.</td>
<td>Pressure washing once every 3 month.</td>
</tr>
<tr>
<td><strong>Disinfectants used</strong></td>
<td>Quatricide PV® (Pharmacal Research Laboratories Inc., Naugatuck, CT) (sanitizing)</td>
<td>Quatricide PV® (sanitizing)</td>
<td>Sodium Hypochlorite (sanitizing)</td>
</tr>
<tr>
<td></td>
<td>Sodium Hypochlorite (pressure washing)</td>
<td>Sodium Hypochlorite (pressure washing)</td>
<td></td>
</tr>
<tr>
<td><strong>Food (brand, type)</strong></td>
<td>Purina Lab Diet</td>
<td>Purina Lab Diet</td>
<td>Hill’s Science Diet Canine Maintenance</td>
</tr>
</tbody>
</table>

Samples (1 gm) were subjected to serial ten-fold dilutions in 0.85% sterile saline solution. For each subsequent dilution, 0.1 ml was transferred onto 3 different media, each in triplicate: MacConkey agar plates for total coliforms count (TCC), and MacConkey agar plates containing either amoxicillin at 16 µg/ml or enrofloxacin at 2
µg/ml for total resistant coliform count (TRCC). The antimicrobial concentrations in the MacConkey agar plates reflected one tube dilution below the respective resistant breakpoint MIC for each drug as set by the Clinical Laboratory Standards Institute (CLSI) for gram negative infections in dogs: 2 mcg/ml for enrofloxacin (breakpoint 4mcg/ml) and 16 mcg/ml for amoxicillin (breakpoint 32 mcg/ml).\textsuperscript{9}

After an 18-24 hr incubation period at 37 °C, the numbers of colony forming units (cfu) were manually determined for each plate. All total colony counts (TCC, and TRCC) were expressed as log\textsubscript{10} cfu per gram wet fecal weight. Antimicrobial resistant coliform counts were expressed as the percent of the total antimicrobial resistant coliform counts to TCC (% RCC = [TRCC/TCC] x100).

\textbf{2.2.3 Statistical Analysis}

Final colony counts for each dog at each sampling time were expressed as the average of the three counts. Total counts were reported as mean ± SE of total log transformed counts at each week for each group. The means of total log transformed coliform counts were compared among and within (across time) groups using PROC MIXED, analysis of variance (ANOVA) and Bonferroni simultaneous confidence intervals with significance at the 0.05 level. Percent resistance to each drug was compared between and within group using a nonparametric method, Flinger-Policello method (SAS Institute Inc.).
2.3 Results

2.3.1 Total coliform counts

A time effect was not detected in TCC for any group (Table 2-2). In contrast, mean TCC was higher (p<0.05) in G1 (6.76 ± 1.20) compared to G2 (5.20 ± 1.05), and G3 (5.61 ± 1.46).

2.3.2 Percent antimicrobial resistance

Time effects in %RCC to amoxicillin and enrofloxacin resistance were detected in G1 only. For resistance to amoxicillin, %RCC approximated 50% for the first 3 weeks but significantly increased to 90% by week 4 (p<0.05) (Figure 2-1a). A time effect in %RCC was also noticed in the two dogs, which joined the study (G1) at the third week of sample collection. For these two dogs, %RCC to amoxicillin increased from 0 to 90%, reaching equality with the other 6 dogs, by week 2 of sample collection (week 4 for remaining G1 dogs). A time effect in the proportion of amoxicillin resistant isolates was not detected in either G2 or G3 (Figures 2-1b and c). A group effect was present for average amoxicillin resistance. Percent RCC to amoxicillin was greatest (p<0.05) in G1 (60.84 ± 38.58) compared to G2 (30.52 ± 35.57) and G3 (5.56 ± 19.67).

For resistance to enrofloxacin, %RCC in G1 significantly increased in week 4, but no significant differences were found in G2 (Figures 2-1a and b). No resistance to enrofloxacin was detected in G3, precluding time effects. A group effect was found in
that %RCC to enrofloxacin differed among the 3 groups, being greatest in G1 (23.81 ± 29.24), less in G2 (7.16 ± 22.81) and least in G3 (0) (p<0.05).

Table 2-2 Weekly mean ± SE of log transformed total coliform counts (TCC) in each group (n=8 per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>B5</th>
<th>B6</th>
<th>AVG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.61±0.39</td>
<td>6.94±0.40</td>
<td>6.29±0.45</td>
<td>6.88±0.60</td>
<td>6.79±0.72</td>
<td>7.16±0.72</td>
<td>6.78±0.21</td>
</tr>
<tr>
<td>2</td>
<td>5.21±0.27</td>
<td>5.24±0.47</td>
<td>4.95±0.47</td>
<td>5.42±0.28</td>
<td>NA</td>
<td>NA</td>
<td>5.20±0.19</td>
</tr>
<tr>
<td>3</td>
<td>6.21±0.46</td>
<td>5.12±0.51</td>
<td>6.07±0.56</td>
<td>5.04±0.48</td>
<td>NA</td>
<td>NA</td>
<td>5.61±0.26</td>
</tr>
</tbody>
</table>

2.4 Discussion

In this study, amoxicillin and enrofloxacin were used to select antimicrobial resistance colonies, in part because this study was a preliminary study in which the appropriate dog source, kennel environment and husbandry needed to be identified before applied to our main study. In addition, the general prevalence of antimicrobial resistance, especially in class B dogs, needed to be investigated before starting the main study. The data from this study would be applied to our main research on the impacts of routine antimicrobial therapy on antimicrobial resistance in fecal Escherichia coli in dogs. Amoxicillin and enrofloxacin will be used as the representative semisynthetic and synthetic antimicrobial drugs respectively.
Figure 2-1 Baseline weekly total coliform counts (–o–), percent amoxicillin resistant coliforms (■), and percent enrofloxacin resistant coliforms (□) in (a) random source, open access dogs (G1; n=8), (b) purpose-bred, limited access dogs (G2; n=8), and (c) purpose-bred, restricted access dogs (G3; n=8).
The lack of significance in higher TCC of G1 probably reflected the small number of dogs with variables in each sample. Nonetheless, TCC in G1 was close to the baseline TCC reported in random, mixed-breed dogs (7.3 ± 1.3 per gram feces). In that study, history on antimicrobial therapy of the dogs was consistent with that of G1 dogs in this study, supporting the premise that uncontrolled exposure of dogs to their surroundings increases the likelihood that fecal coliform resistance might emerge.

From our data, the percent of isolates resistant to amoxicillin was markedly variable between and within groups, resulting from variation between individual dogs. In general, the percent coliforms resistant to amoxicillin in G1 were significantly higher from the other 2 groups. In addition, no significant differences (p>0.05) were found between G2 and G3, suggesting an impact of dog source, with amoxicillin resistance being less in purpose-bred dogs (G2 and G3). In G1, resistance to amoxicillin significantly increased in 4th week of study, suggesting the transmission of resistance between the dogs. Resistance to neither amoxicillin nor enrofloxacin was evident in either of these two dogs for the first week of their residence in the new environment. However, resistance emerged and rapidly increased to 90% in the 2nd week of transfer, about the same time amoxicillin resistance dramatically increased (>90%) in the other four G1 dogs. This is suggestive of a mechanical transfer of amoxicillin resistant genes among dogs in this kennel.

The pattern of percent enrofloxacin resistant coliforms in G1, although similar to that of amoxicillin, was overall less in magnitude. It is possible that amoxicillin resistance more easily emerges in dogs compared to enrofloxacin because of enteric
exposure to antibiotics produced by enteric microbiota. Amoxicillin is a semi-synthetic penicillin, whereas enrofloxacin is a synthetic drug. Mechanisms for resistance may more easily emerge if microbes are normally exposed to the compound. As a semi-synthetic, enteric microbes produce amoxicillin but none produce synthetic quinolones. Thus enteric microbes are likely to be exposed to amoxicillin but not to enrofloxacin. In addition, the mechanisms of resistance to amoxicillin and enrofloxacin are different: amoxicillin resistance is generally horizontally transmitted via plasmids, whereas enrofloxacin resistance is generally through mutations of the chromosomal DNA. Therefore amoxicillin resistant genes may be faster transmitted among the dogs compared to enrofloxacin resistant genes.

In G2, with the limited access to the kennel, amoxicillin resistance was moderate. However, some variability occurred among dogs in this group. Three out of eight dogs in this group had no amoxicillin resistance up to and during the first four weeks of data collection, whereas the other 5 dogs were characterized by moderate resistance (35-80%) to amoxicillin (data not shown). For enrofloxacin resistance, only two dogs expressed enrofloxacin resistant coliforms (65-90%) for only a total of 3 time-points between the two dogs. However, those enrofloxacin resistant isolates rapidly disappeared within 7 days. The pattern of resistance emergence and resolution suggests that mechanical transmission of resistance genes did not occur in this group.

In G3, fecal coliforms expressed very low amoxicillin resistant coliforms (0-30%), presumably reflecting the strictly controlled environment, thus supporting the impact of kennel environment on fecal amoxicillin resistance coliforms. No G3 dog
expressed resistance to enrofloxacin. This suggests that not only the kennel environment, but also the source of the dogs (i.e. non-purpose bred with unknown history versus purpose-bred) impacts the presence of fecal antimicrobial resistance coliforms in dogs.

Although this study did not identify the fecal coliforms in which resistance was detected, *Escherichia coli* is the predominant Gram negative facultative anaerobe isolated from feces of dogs.² It also is the fecal gram negative coliform most commonly associated with infections and is among the most frequently reported antimicrobial resistant enterobacteria among fecal coliforms.² The prevalence of antimicrobial resistance *E. coli* in dogs is reportedly more common in kennel dogs, in contrast to the individually owned dogs, probably due to group housing¹⁷ in which the horizontal transfer of the resistance genes occur rapidly. The prevalence of antimicrobial resistance was recently reported in fecal *E. coli* in the healthy, individually owned dogs in Europe.¹⁸ Our study, however, has demonstrated that the prevalence of antimicrobial resistance in fecal *E. coli* in healthy, group housing dogs was reduced or not present in the purpose-bred dogs with the restricted control kennel that restricted direct contacts to other animals. Therefore, the kennel environment and dog source impact fecal coliform antimicrobial resistance.

2.5 Conclusion

This study demonstrated that the percent of antimicrobial resistant fecal coliforms was higher in random source dogs, lesser in open access purpose bred dogs and least in restricted access purpose bred dogs. These findings strongly suggest that kennel
environment and dog source have significant impact on antimicrobial resistance of fecal coliforms. However, their specific roles in this matter have yet to be determined.

2.6 References


3.1 Introduction

Emergence of antimicrobial resistance is an increasing concern in both human and veterinary medicine.\textsuperscript{1,2} Although not as critical as those reported in human medicine, antimicrobial resistance in canine medicine has been reported. Increased prevalence of antimicrobial resistance in various bacterial species from pet animals has been reported in the United States and the United Kingdom, mainly caused by antimicrobial therapy.\textsuperscript{1-5} The role of previous antimicrobial therapy in the emergence of resistance is generally accepted, as is the role of selection pressure.\textsuperscript{6} Use of antimicrobials not only selects for resistance in the pathogenic bacteria, but also in commensal bacteria.\textsuperscript{5-8}

However, the science behind this acceptance is limited and, as such, so is our understanding of the mechanisms. This lack of understanding curtails our ability to design and implement protocols that might minimize both emergence and clinical impact of resistance. Although a number of animal models have been developed to investigate antimicrobial-induced resistance, little has been done in companion animals. The long-term objective of our laboratory is to understand the role of routine antimicrobial
therapy in the emergence of resistant microorganisms in the patient, the role such emergence subsequently has on patient’s health, and to identify interventions which might minimize the advent and impact of resistance on animal or public health.

*Escherichia coli* is a member of the family Enterobacteriaceae, and a common inhabitant of the intestinal tracts of animals and humans among the gastrointestinal commensal flora.9-10 Fecal *E. coli* is frequently used to represent the intestinal flora due to the ease and convenience of sample collection in live animals.7, 11 In addition, fecal *E. coli* is considered as a very good indicator for selection pressure by antimicrobial use.7

Amoxicillin and enrofloxacin are among the most commonly used antimicrobials in veterinary medicine. Amoxicillin is a semi-synthetic β-lactam, while enrofloxacin is a synthetic fluoroquinolone (FQ).12-15 The purpose of this study was to conduct a preliminary investigation into the routine use of popular antimicrobials with the advent of antimicrobial resistance in dogs, using fecal coliforms and especially, *E. coli*, as sentinel organism.

### 3.2 Materials and Methods

#### 3.2.1 Animals and sample collections

Six healthy, antimicrobial-free, purpose-bred adult hound dogs were randomly divided in three groups of two. Each group received different antimicrobial therapy: group1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 h, group2 (G2) was treated with 5 mg/kg enrofloxacin orally every 24 h, and Group3 (G3) received no
treatment and was reserved as a control group. Both drugs were administered for 7 days. All dogs were maintained with regular adult maintenance diet and each dog was randomly housed in individual, climate controlled cage in close proximity to each other with a restricted access to the kennel to minimize mechanical transmission of microbes and antimicrobial resistance genes.

3.2.2 Total and resistant coliform counts

Fresh fecal samples were collected per rectum into sterile containers prior to (D0), days (D) 1, 3, 5, 7 of treatment, and days (P) 3, 7, 14 and 21 post-therapy. All samples were collected on the same time of each sampling day, within 1 h post-feeding. Fecal samples were processed within 2 h of collection. Serial 10-fold dilutions were prepared from 1 g fecal sample in 0.9% sodium chloride solution. For each subsequent dilution, 0.1 ml was transferred onto MacConkey agar plates or plates supplemented with either amoxicillin or enrofloxacin at concentrations one tube dilution below the respective resistant breakpoint MIC (MICBP) for each drug as set by the Clinical Laboratory Standards Institute (CLSI) for gram negative bacteria in Veterinary Medicine: 2 µg/ml for enrofloxacin (MICBP 4 µg/ml) and 16 µg/ml for amoxicillin (MICBP 32 µg/ml), for total coliforms counts (TCC) and for total resistant coliform counts (TRCC), respectively. After an 18 to 24 hour incubation period at 37 °C, the numbers of colony forming units (cfu) were manually determined from each plate. All samples were performed in triplicate with the final counts expressed as the mean of the three counts. Both TCC and TRCC of each drug were expressed as log_{10} cfu per gram wet fecal
weight. Antimicrobial resistant coliform counts were expressed as the proportion of TRCC of each drug to TCC ([TRCC/TCC] x100).

3.2.3 *E. coli* identification and level of antimicrobial resistance

Generic *E. coli* colonies appear as pink to brick-red colonies with or without a zone of precipitated bile on MacConkey agar. Based on morphology, 10 *E. coli* colonies resistant to treatment drug at each time-point were randomly selected from antimicrobial containing MacConkey agar plates and confirmed by *E. coli* screen with Kovacs tests (Remel/ Thermo Fisher Scientific, Lenexa, KS) before susceptibility tests to amoxicillin and enrofloxacin were performed using Etest® (Epsilometer or Epsilon; AB Biodisk/ BioMérieux Inc., Hazelwood, MO) strips according to the manufacturer’s instructions. Briefly, each 18 to 24 h growth isolate was adjusted to a McFarland standard of 0.5 in 0.9% sodium chloride solution. Each inoculum was saturated onto a swab and inoculated by confluent swabbing of the surface onto a Mueller-Hinton agar plate. Inoculated plates were allowed to dry before amoxicillin and enrofloxacin Etest strips were applied to the medium. The antimicrobial concentration ranges determined on Etest strips were 0.016 to 256 µg/ml for amoxicillin, and 0.002 to 32 µg/ml for enrofloxacin. All plates were incubated at 37°C for 18 to 24 h. The results (MICs) were determined on the basis of the intersection of the elliptical zone of growth inhibition with the MIC scale on each Etest strip and expressed as the MIC required to inhibit the growth of 90% of the isolates (MIC$_{90}$). MIC$_{BP}$ of amoxicillin and enrofloxacin are ≥ 32 and ≥ 4 respectively.
3.2.4 Type of antimicrobial resistance

Five out of each 10 antimicrobial resistant isolates determined for the level of resistance were randomly selected and determined for susceptibility using Vitek® automated system (BioMerieux Inc., Hazelwood, MO) with Gram Negative Veterinary Susceptibility Test Cards (GNS-207). MICs were determined based on susceptibility to 17 antimicrobial drugs with 8 drug classes (Table 3-1); Amikacin, Amoxicillin/clavulanic acid, Ampicillin, Carbenicillin, Ceftazidime, Ceftiofur, Cephalothin, Chloramphenicol, Ciprofloxacin, Enrofloxacin, Gentamicin, Nitrofurantoin, Piperacillin, Tetracycline, Ticarcillin, Tobramycin, and Trimethoprim/Sulfamethoxazole according to the manufacturer’s instructions. Briefly, each isolate was grown for 18 to 24 h before being adjusted to a McFarland standard of 1 in 0.45% sodium chloride solution using Vitek® DensiCheck (BioMerieux Inc., Hazelwood, MO). Then 50 μl of the suspension was added to 2 ml of 0.45% sodium chloride solution to make a working suspension. Each test card was filled up with the working suspension using vacuum before being loaded into the Vitek® machine. Results were analyzed and phenotypes of each resistant E. coli colony were expressed as susceptible (S) or resistant (R) based on CLSI MIC_{BP} of E. coli to each drug (M31-S1). Presence of multidrug resistance (MDR) was determined based on resistance to 3 or more unrelated drug classes.
Table 3-1 Antimicrobial drugs, drug classes and concentrations (µg/ml) determined on Vitek® Gram Negative Veterinary Susceptibility Test Cards (GNS-207) and MIC\textsubscript{BP} (µg/ml) for resistance of each drug based on CLSI guideline (M31-S1)\textsuperscript{14}

<table>
<thead>
<tr>
<th>Drug classes</th>
<th>Antimicrobial drugs</th>
<th>Concentrations</th>
<th>MIC\textsubscript{BP}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Ampicillin (A)</td>
<td>0.5, 4, 32</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin/Clavulanic acid (X)</td>
<td>4/2, 8/4, 18/8</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Carbenicillin (B)</td>
<td>32, 256</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td>Piperacillin (P)</td>
<td>8, 32, 64</td>
<td>≥ 256</td>
</tr>
<tr>
<td></td>
<td>Ticarcillin (R)</td>
<td>32, 64, 128</td>
<td>≥ 256</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Ceftazidime (Z)</td>
<td>4, 8, 64</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Ceftiofur (C)</td>
<td>2, 4, 8</td>
<td>≥ 8</td>
</tr>
<tr>
<td></td>
<td>Cephalothin (L)</td>
<td>4, 16</td>
<td>≥ 32</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin (F)</td>
<td>1, 4</td>
<td>≥ 4</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacin (E)</td>
<td>0.25, 0.5, 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Amikacin (K)</td>
<td>2, 8, 32</td>
<td>≥ 64</td>
</tr>
<tr>
<td></td>
<td>Gentamicin (G)</td>
<td>0.5, 2, 8</td>
<td>≥ 16</td>
</tr>
<tr>
<td></td>
<td>Tobramycin (M)</td>
<td>0.5, 2, 8</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline (T)</td>
<td>2, 8, 32</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Phenicols</td>
<td>Chloramphenicol (H)</td>
<td>1, 8</td>
<td>≥ 32</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Trimethoprim/Sulfamethoxazole (S)</td>
<td>2/38, 8/152</td>
<td>≥ 4/76</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Nitrofurantoin (N)</td>
<td>32</td>
<td>≥ 128</td>
</tr>
</tbody>
</table>

3.2.5 Pulse-field gel electrophoresis (PFGE)

Three representative isolates were randomly selected from antimicrobial resistant \textit{E. coli} of each phenotype from each group, and a total of 21 isolates, were characterized using PFGE in accordance with the Pulse-Net standardized protocol for molecular
subtyping of \textit{E. coli} O157:H7.\textsuperscript{22} Antimicrobial resistant isolates were grown for 14 to 18 hours before being suspended in cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0). Concentrations of cell suspensions were adjusted to the absorbance of 1.3-1.4 at 610 nm wavelength in a spectrophotometer. Plugs were prepared by mixing 400 µl of cell suspension, 20 µl of a 2% proteinase K solution, and an equal volume of 1% SeaKem Gold:1% sodium dodecyl sulfate (SDS) agarose and dispensed into reusable plug molds. Plugs were allowed to solidify at room temperature for 10-15 minutes before being removed from the molds. Cells embedded in plugs were lysed in cell lysis buffer containing 20 mg/ml proteinase K in a shaker water bath at 54°C, 150-175 rpm for 1.5-2 h. Lysed plugs were washed with preheated sterile ultrapure water and TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) respectively. Plugs were then digested with a restriction enzyme \textit{XbaI} (50 U/plug) and resolved on a 1% SeaKem Gold Agarose gels in 0.5×Tris-Borate EDTA Buffer at 14°C. The running conditions for the PFGE gels were: initial switch time 5 s; final switch time 40 s; duration of run 18 h, angle, 120°; gradient, 6 V/cm with a linear ramping factor using a CHEF MAPPERS II System (Bio-Rad, Hercules, CA). Pattern images were acquired by staining gels with ethidium bromide at a final concentration of 10 µg/ml. \textit{Salmonella enterica} serovar Braenderup H9812 was used as the size standard strain.
3.2.6 Data Analysis

Final colony counts for each dog at each sample collection time were expressed as the average of three counts. Total counts were reported as mean ± SE of total log transformed counts at each time-point for each group.

For PFGE analysis, Tiff images of the gels were normalized using the BioNumerics® software, version 4.5 (Applied Maths, Austin, TX). Analysis of band patterns and construction of dendrograms were performed using the Dice correlation coefficient and clustering of patterns was performed by unweighted pair group with arithmetic averaging (UPGMA). Ninety percent of similarity between patterns was used to address the relatedness among the PFGE patterns.23

3.3 Results

3.3.1 Total coliform counts

Mean TCC did not differ across time within or among the treatment groups (Table 3-2). TCCs were $10^4 - 10^6$, $10^5 - 10^7$, and $10^5 - 10^6$ cells per gram feces in G1, G2 and G3 respectively. Most (>90%) of resistant coliforms were *E. coli* based on morphology.
Table 3-2 Mean ± SE of log transformed total coliform counts (TCC) in each group (n=2 per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>D0</th>
<th>D1</th>
<th>D3</th>
<th>D5</th>
<th>D7</th>
<th>P3</th>
<th>P7</th>
<th>P14</th>
<th>P21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.8±0.49</td>
<td>5.79±0.59</td>
<td>5.54±0.21</td>
<td>5.69±0.58</td>
<td>6.37±0.07</td>
<td>5.96±0.27</td>
<td>5.91</td>
<td>5.13±0.57</td>
<td>5.4±0.51</td>
</tr>
<tr>
<td>2</td>
<td>4.21±1.03</td>
<td>5.24±1.41</td>
<td>5.89±0.58</td>
<td>4.88±0.67</td>
<td>4.3±0.53</td>
<td>5.73±0.12</td>
<td>5.53±1.11</td>
<td>5.35±0.31</td>
<td>5.04</td>
</tr>
<tr>
<td>3</td>
<td>5.19±1.55</td>
<td>5.29</td>
<td>4.81±0.29</td>
<td>5.9±0.08</td>
<td>7±0.25</td>
<td>6.29±1.13</td>
<td>5.6±0.5</td>
<td>5.55±1.11</td>
<td>5.85±0.52</td>
</tr>
</tbody>
</table>

3.3.2 Percent antimicrobial resistance

3.3.2.1 Resistance to amoxicillin

Twenty-five percents of coliforms were resistant to amoxicillin at D0 in G1, while no resistance was detected at D0 in G2 and G3 (Figure 3-1). In G1, resistance to amoxicillin gradually increased to close to 100% by D3 and resolved by P21. In G2, amoxicillin resistance emerged by D3 but remained throughout the study. In G3, amoxicillin resistance was transiently present at D5 and D7.

3.3.2.2 Resistance to enrofloxacin

No enrofloxacin resistance was detected at D0 in any group at any time point (Figure 3-1). In G1, resistance to enrofloxacin was transiently present at D3 and D5. In G2, enrofloxacin resistance emerged by D3 but persisted throughout the study. No enrofloxacin resistance was present in any time-point in G3 dogs.
Figure 3-1 Total coliform counts (○), percent amoxicillin resistant coliforms (■), and percent enrofloxacin resistant coliforms (□) in (a) random source, open access dogs (G1; n=2), (b) purpose-bred, limited access dogs (G2; n=2), and (c) purpose-bred, restricted access dogs (G3; n=2).
3.3.3 *E. coli* screen, level and type of antimicrobial resistance

All antimicrobial resistant coliform colonies randomly subjected to *E. coli* screen were *E. coli*. For G1 isolates, MIC90 for amoxicillin was ≥512 µg/ml whereas the MIC90 for enrofloxacin was 0.125. Amoxicillin-induced resistance was associated with 4 phenotypes, but generally was not MDR unless enrofloxacin resistance was present (Table 3-3). For G2 isolates, MIC90 for enrofloxacin was ≥ 64 µg/ml and toward amoxicillin ≥ 512 µg/ml. Enrofloxacin-induced resistance yielded 2 phenotypes and generally exhibited MDR.

3.3.4 PFGE fingerprints

Among 21 representative isolates subjected to PFGE, genomic fingerprinting and dendrogram revealed 90% relationship among amoxicillin-induced amoxicillin resistant, enrofloxacin susceptible isolates (G1) (Fig. 3-2). Enrofloxacin resistant isolates (whether G1 or G2) were less than 90% similarity.
Table 3-3 Phenotypes, frequency, and MIC 90 of antimicrobial resistant *E. coli* from dogs treated with either amoxicillin (G1), enrofloxacin (G2), or no treatment (G3)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time</th>
<th>Phenotypes(^a)</th>
<th>Frequency</th>
<th>MDR(^b)</th>
<th>MIC90 (Etest)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amoxicillin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Enrofloxacin</td>
</tr>
<tr>
<td>G1</td>
<td>Amoxicillin</td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>25%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>50%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLFETSH</td>
<td>25%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>25%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>25%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLFETSH</td>
<td>50%</td>
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<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td>G2</td>
<td>Enrofloxacin</td>
<td></td>
<td>ABPRLFETSH</td>
<td>10%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>90%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLFETSH</td>
<td>60%</td>
<td>Y</td>
<td>≥ 512</td>
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<td></td>
<td>ABPRXLFETSH</td>
<td>40%</td>
<td>Y</td>
<td>≥ 512</td>
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<td>20%</td>
<td>Y</td>
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<tr>
<td></td>
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<td></td>
<td>ABPRXLFETSH</td>
<td>80%</td>
<td>Y</td>
<td>≥ 512</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>10%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>90%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>50%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>50%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>40%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>60%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>80%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETSH</td>
<td>20%</td>
<td>Y</td>
<td>≥ 512</td>
</tr>
<tr>
<td>G3</td>
<td>None (Control)</td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLS</td>
<td>100%</td>
<td>N</td>
<td>≥ 512</td>
</tr>
</tbody>
</table>

\(^a\) A = ampicillin; B = Carbenicillin; P = piperacillin; R = Ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/Sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin

\(^b\) MDR is defined as resistance to 3 or more unrelated drug classes
3.4 Discussion

This pilot study was performed in purpose-bred dogs, housed in a restricted kennel to minimized transmission of microbes and antimicrobial resistance genes. However, transient amoxicillin resistance at D5 and D7 in control dogs (G3) and enrofloxacin resistance at D3 and D5 in amoxicillin-treated dogs (G1) were still present, potentially from mechanical transfer of resistance genes between dogs. Our data demonstrated that neither amoxicillin nor enrofloxacin therapy had direct effects on the total coliforms counts, with *E. coli* being the major coliform in dog feces. These findings disagree with other studies, in which FQ had profound impacts on aerobic population of the gastrointestinal flora by transiently suppressing or eliminating them. Microbes...
returned to pre-antimicrobial administration numbers within 2 weeks after cessation of therapy. Trott et al (2004) showed that dogs given a daily oral enrofloxacin at 5 mg/kg for 21 consecutive days exhibited a significant decline in fecal coliforms to undetectable levels by 3 days of therapy and remained suppressed throughout the period of enrofloxacin dosing. Upon termination of enrofloxacin treatment, fecal coliforms gradually returned to levels comparable to those seen prior to antibiotic treatment by 8 days. This could partly be due to the high variations in numbers of fecal coliforms from our triplicate plates per sampling time and the small sample size (n=2 dogs per group) in our study. However, the proportions of antimicrobial resistance in G1 and G2 dogs were different, in response to either amoxicillin or enrofloxacin treatment, compared to G3 (no treatment) dogs.

In amoxicillin-treated dogs, amoxicillin resistance rapidly developed in both dogs by 3 days of therapy and resolved by 3 week post-treatment. Although enrofloxacin resistance was detected in both dogs on day 3 and day 5, it was less likely to be due to amoxicillin therapy as it rapidly disappeared before amoxicillin treatment was discontinued (while amoxicillin resistance was still close to 100%). In addition, the 2 time-points that G1 dogs developed resistant to enrofloxacin were about the same time the dogs in G2 (enrofloxacin-treated) rapidly developed high (close to 100%) resistance to enrofloxacin. Therefore, it is possible that enrofloxacin resistance in these amoxicillin-treated dogs was due to the mechanical transfer of resistance genes between G2 and G1 dogs.
For dogs treated with enrofloxacin, not only enrofloxacin resistance but also amoxicillin resistance developed by day 3 of enrofloxacin therapy. However, neither enrofloxacin resistance nor amoxicillin resistance resolved, at least for 3 weeks post-therapy. This indicates the different mechanism of resistance, especially amoxicillin resistance, mediated by enrofloxacin treatment from that mediated by amoxicillin resistance. More importantly, enrofloxacin induced resistance to multiple antimicrobials in which their mechanisms of resistance are different from that of enrofloxacin.

Enrofloxacin is a veterinary FQ whose resistance is mainly due to mutations in bacterial *gyrA* and *parC* genes that code for DNA gyrase and topoisomerase IV enzymes. Moreover, decrease of FQ concentration in bacterial cell via efflux pumps such as AcrAB-TolC efflux system also plays an important role for FQ-mediated MDR in *E. coli*. This efflux system mediates resistance to several drugs including FQ, ampicillin, tetracycline, chloramphenicol, rifampicin, and puromycin. Horizontal transfer of FQ resistance genes by either plasmids or other mobile DNA, such as transposons which involves the transfer of FQ resistant efflux pump genes has recently been reported as another mechanism of FQ resistance. This horizontal transfer provides the genetic linkage between resistance to FQ and β-lactam drugs. Moreover, plasmid transfer of FQ resistance genes may explain the rapid increase of FQ resistance, as well as high-level of FQ resistance in addition to mutations in bacterial DNA gyrase and/or topoisomerase IV enzymes.

On the other hand, amoxicillin mediated non-MDR in fecal *E. coli* in this study. This could be due to the predominant mechanism of amoxicillin resistance in Gram-
negative bacteria, the production of β-lactamases. Beta-lactamases are the specific enzymes which are encoded either chromosomally or by plasmids and inactivate β-lactams by hydrolyzing the four-membered β-lactam rings.\textsuperscript{37-39} However, high-level resistance to sulfamethoxazole/trimethoprim, but not other drugs, was noticed in the majority (>90%) of these non-MDR isolates. This indicates the involvement of either a resistance mechanism which is more specific to the sulfamethoxazole/trimethoprim resistance genes such as chromosomal mutations that cause an overexpression of the host substrate, or the acquisition of a gene encoding a resistant enzyme to sulfonamides and/or trimethoprim by mobile DNA such as plasmids, integrons and transposons.\textsuperscript{40-42} Acquisition of resistance enzymes horizontally cause high-level resistance to either sulfonamides or trimethoprim, or both, supporting our findings in this study.

3.5 Conclusion

The pilot data presented here suggest that either amoxicillin or enrofloxacin when administered at recommended dosing regimens is associated with rapid development of high level antimicrobial resistance to that drug in fecal coliform, especially \textit{E. coli}. Further, the behavior differs in that resistance associated with amoxicillin resolves when therapy is discontinued while the resistance to enrofloxacin persists. Finally, and most disconcertingly, resistance associated with enrofloxacin, but not amoxicillin, is MDR.
3.6 References


CHAPTER 4

MULTIDRUG RESISTANCE IN FECAL *Escherichia coli* FOLLOWING ROUTINE
ENROFLOXACIN BUT NOT AMOXICILLIN THERAPY IN DOGS

4.1 Introduction

Emergence of antimicrobial resistance in response to routine antimicrobial
erapy, especially fluoroquinolones (FQ), is of great concern in both human and
veterinary medicine.\(^1\)\(^6\) Not only does it exert a selection pressure on pathogenic bacteria,
antimicrobial therapy also affects intestinal commensal bacteria in both humans and
animals, and promotes colonization with more resistance bacteria.\(^2\)\(^3\)\(^7\)\(^14\) Moreover,
animal intestine is an ideal environment for the selection and transfer of antimicrobial
resistance genes.\(^15\)\(^16\) However, antimicrobial-induced resistance is generally based on the
clinical studies that associate drug use with increasing rates of resistance in population
rather than measuring resistance in experimentally-induced resistance in individual
patient.\(^12\) In addition, it is difficult to isolate several species of bacteria in the
gastrointestinal tract as many of them are present in low numbers and many of them
cannot be cultivated.\(^11\)
Among the normal flora of gastrointestinal tract, *Escherichia coli* is a common inhabitant of the intestinal tracts of animals and humans.\textsuperscript{17-18} Although little is known about the distribution of resistance in rectal *E. coli* strains from companion animals, fecal *E. coli* is frequently used as representative of the enterobacteria in the intestinal flora, partly because of the ease and convenience of sample collection in live animals compared to collecting samples from other parts of gastrointestinal tract.\textsuperscript{11-12, 19} More importantly, fecal *E. coli* is considered as a very good indicator for selection pressure by antimicrobial use.\textsuperscript{11} *E. coli* of healthy pets have also been reported as a reservoir of antimicrobial resistance genes.\textsuperscript{12, 14, 19} *E. coli* is among the organisms that have developed resistance to the fluorinated quinolones, beta-lactams or both.\textsuperscript{20-23}

This study was aimed to associate the routine use of the two popular antimicrobials, amoxicillin and enrofloxacin, with the advent of resistance in the animal, using fecal *E. coli* as the sentinel organism. Amoxicillin and enrofloxacin are among the most commonly used antimicrobials in veterinary medicine. Amoxicillin is a semi-synthetic β-lactam to which the microbes may naturally be exposed, compared to enrofloxacin, which is a synthetic fluoroquinolone drug.

Our pilot data suggest that either amoxicillin or enrofloxacin therapy at the recommended dosing regimens for 7 days mediates the high level resistance to the treatment drug in fecal coliforms, especially *E. coli*. The resistance patterns differ in that amoxicillin treatment is associated with non-multidrug (non-MDR) resistance whereas enrofloxacin resistance is associated with MDR. Moreover, resistance behavior to either
drug is different because resistance to amoxicillin resolves when therapy is discontinued, while resistance to enrofloxacin persists.\textsuperscript{34}

In this study, we compared the impact of routine use of these two drugs on fecal \textit{E. coli}, a major flora in the gastrointestinal tract in dogs, in the terms of emergence of resistance, time to resistance and time to resolution of the resistance, as well as level and type of resistance.

\section*{4.2 Materials and Methods}

\subsection*{4.2.1 Animals and sample collections}

Twenty-four healthy, antimicrobial-free, purpose-bred adult hound dogs were studied. All dogs were maintained with regular adult maintenance diet (Hill’s\textsuperscript{®} Science Diet\textsuperscript{®} Adult Large Breed dry food, Hill’s Pet Nutrition Inc., Topeka, KS). Each dog was randomly housed in individual, climate controlled cage in a strictly-controlled environment, with an empty cage between each dog to prevent dog-to-dog contact, as well as kennel access limitation, and weekly disinfection to minimize mechanical transmission of microbes and antimicrobial resistance genes.

Dogs were randomly divided in three groups of eight. Each group received a different antimicrobial therapy: group1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 h, group2 (G2) was treated with 5 mg/kg enrofloxacin orally every 24 h, and Group3 (G3) received no treatment and was reserved as a control group. Both drugs were administered for 7 to 21 days, with the duration based on the emergence of
antimicrobial resistance, defined as a proportion of total resistant *E. coli* to total *E. coli* counts ≥75%. The time at ≥75% resistance was defined as time of resistance (T), and then antimicrobial therapy was discontinued.

Fresh fecal samples were digitally collected *per rectum* into sterile containers (VWR International, West Chester, PA) prior to (Baseline; B) and every 3 days during treatment until time of resistance (T). Drug was then discontinued and monitoring continued weekly up to 4 weeks, or until the resistance resolved (≤25% of cfu resistant or study end; E), whichever came first. If the resistance did not resolve by 4 weeks post-therapy, E would be reported as 4 weeks.

### 4.2.2 Total and resistant coliform and *E. coli* counts

Samples were collected on the same time of each sampling day, within 1 h post-feeding. Fecal samples were processed within 2 h of collection. Serial 10-fold dilutions were prepared from 1 g fecal sample in 0.9% sodium chloride solution. Total coliform counts (TCC) and total *E. coli* counts (TEC) were obtained by transferring 0.1 ml of the appropriate dilutions in onto the surface of MacConkey and CHROMagar® *E. coli* agar plates respectively (CHROMagar, Paris, France). CHROMagar® *E. coli* agar is a culture medium for detection and enumeration of *E. coli* based on the presence of β-glucuronidase enzyme. Total amoxicillin and enrofloxacin-resistant *E. coli* (TREC) were obtained by transferring 0.1 ml of the appropriate dilutions onto the CHROMagar® *E. coli* plates containing either amoxicillin or enrofloxacin at concentrations 1 tube
dilution below the breakpoint MIC (discriminatory antimicrobial concentrations used in the interpretation of results of susceptibility testing to define isolates as susceptible, intermediate or resistant; MICBP) of either drug: at 16 µg/ml agar for amoxicillin and 2 µg/ml agar for enrofloxacin respectively. Plates were incubated at 37°C for 18 to 24 h. All samples were performed in triplicate with the final counts expressed as the mean of the three counts.

Percent E. coli in feces (%EC) and percent resistant E. coli to either drug (%REC) were expressed as the proportions of the TEC to TCC ([TEC/TCC] x100) and those of the TREC to TEC ([TREC/TEC] x100) consecutively.

4.2.3 Level of antimicrobial resistance

Ten E. coli colonies resistant to each treatment drug from each dog at each time-point were randomly selected from CHROMagar E. coli plates containing either amoxicillin drug as described in 4.2.2. Susceptibility to amoxicillin and enrofloxacin was performed using Etest® (Epsilometer or Epsilon; AB Biodisk/ BioMérieux, Inc. Hazelwood, MO) strips according to the manufacturer’s instructions. Briefly, each 18 to 24 h growth isolate was adjusted to a McFarland turbidity standard of 0.5 in 0.9% sodium chloride solution. Each inoculum was saturated onto a swab and inoculated by confluent swabbing of the surface onto a Mueller-Hinton agar plate. Inoculated plates were allowed to dry before amoxicillin and enrofloxacin Etest strips were applied to the medium. The antimicrobial concentration ranges determined on Etest strips were 0.016 to 256 µg/ml
for amoxicillin, and 0.002 to 32 µg/ml for enrofloxacin. All plates were incubated at 37°C for 18 to 24 h. The results (MICs) were determined on each isolate on the basis of the intersection of the elliptical zone of growth inhibition with the MIC scale on each Etest strip and expressed as the MIC required to inhibit the growth of 90% of the isolates (MIC$_{90}$). Level of resistance was based on MIC$_{BP}$ of either drug for the; ≥ 32 for amoxicillin and ≥ 4 for enrofloxacin respectively.

### 4.2.4 Type of antimicrobial resistance

All isolates determined for the level of resistance were further determined for susceptibility using Vitek® automated system (BioMérieux, Inc. Hazelwood, MO) with Gram Negative Veterinary Susceptibility Test Cards (GNS-207). MICs were determined based on susceptibility to 17 antimicrobial drugs, representing 8 drug classes (Table 4-1); Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Carbenicillin, Ceftazidime, Ceftiofur, Cephalothin, Chloramphenicol, Ciprofloxacin, Enrofloxacin, Gentamicin, Nitrofurantoin, Piperacillin, Tetracycline, Ticarcillin, Tobramycin, and Trimethoprim/Sulfamethoxazole according to the manufacturer’s instructions. Briefly, each isolate was grown for 18 to 24 h before being adjusted to a McFarland standard of 1 in 0.45% sodium chloride solution using Vitek® DensiCheck (BioMérieux, Inc. Hazelwood, MO). Then 50 µl of the suspension was added to 2 ml of 0.45% sodium chloride solution to make a working suspension. Each test card was filled up with the working suspension using vacuum before being loaded into the Vitek® machine. Results were analyzed and phenotypes of each resistant *E. coli* colony were expressed as
susceptible (S) or resistant (R) based on CLSI MIC\textsubscript{BP} of \emph{E. coli} to each drug (M31-S1).\textsuperscript{27}

Presence of multidrug resistance (MDR) was indicated if \emph{E. coli} was resistance to 3 or more unrelated drug classes.\textsuperscript{28}

\subsection*{4.2.5 Statistical Analysis}

Final colony counts for each dog at each sample collection time were expressed as the average of the three counts. TCC, TEC and TREC were reported as mean ± SE of total log transformed counts at each time-point for each group. All the results were compared between treatments and time-points by Standard One-way Analysis of Variance (ANOVA) with Dunnett’s multiple comparisons (JMP® 7.0.2 software, SAS Institute Inc.).

\section*{4.3 Results}

\subsection*{4.3.1 Total and proportional counts}

In G1, TCC and TEC differed significantly between B and T (p=0.0155 and p=0.0095 respectively), but not between B and E, or T and E (Figure 4-1A). Significant differences also were not detected between %EC in G1 dogs at any time-point. In contrast, for G2, neither TCC nor TEC differed across time but significantly differed in %EC between B and T (p=0.0015) (Figure 4-1B). Interestingly, \emph{E. coli} in 4 dogs of G2 was undetectable by day 9 of therapy and remained undetectable post-treatment, towards
the end of study. No significant differences were detected among TCC, TEC and %EC in G3 (Figure 4-1C).

When compared between treatments, neither TCC nor TEC differed at each time-point. However, %EC statistically differed between G1 and G2 at T (p=0.0003), but not at B or E time-points.

4.3.2 Time periods and proportion of resistance

In G1, time to amoxicillin resistance was 6.67±0.83 days with %REC = 92.71±2.97. Resistance returned to baseline by 1.13±0.13 weeks (7.88±0.88 days) after therapy was discontinued. Percent REC to amoxicillin differed significantly between B and T, and T and E (p<0.0001) (Table 4-2). One out of 8 dogs in G1 also expressed enrofloxacin resistance with %REC = 0.66 by day 9, and resolved by 1 week post-therapy.

In G2, time to enrofloxacin resistance was 9 days with %REC = 103.64±1.71. However, no E. coli was detected in the other 4 of G2 dogs post-therapy, precluding assessment of E coli resistance in those dogs (Figure 4-2). Percent REC to enrofloxacin differed significantly between B and T, and T and E (p<0.0001 and p<0.0002 respectively), but not between B and E. Enrofloxacin resistance resolved in 3 out of 4 dogs by 2.25±0.75 weeks (15.75±5.25 days) after enrofloxacin was discontinued. G2 isolates also expressed resistance to amoxicillin with %REC = 88.17±12.81 by day 9 as in the presence of enrofloxacin resistance. Amoxicillin resistance in G2 resolved by
1.25±0.25 weeks (8.75±1.75 days) post-therapy. There is no difference between time to enrofloxacin resolution and time to amoxicillin resolution. Significant differences were detected in %REC to amoxicillin between B and T, and T and E (p<0.0001). No amoxicillin or enrofloxacin resistance was present at time B for any group, as well as in G3 dogs.

No differences were detected among time to amoxicillin resistance, time to amoxicillin resolution, and %REC to amoxicillin between G1 and G2 at any time-point. However, %REC to enrofloxacin significantly differed between G1 and G2 at T (p<0.0004), but not at B or E time-points.

4.3.3 Level and type of antimicrobial resistance

Amoxicillin resistant E. coli in G1 exhibited high-level resistance to amoxicillin, with similar MIC25, MIC50 and MIC90 (≥ 256) but susceptible to enrofloxacin (Table 4-3). However, enrofloxacin resistant E. coli isolates received from 1 dog in G1 exhibited high-level resistant to both amoxicillin and enrofloxacin. In G2, enrofloxacin resistant E. coli exhibited high-level resistance to both enrofloxacin and amoxicillin. For G3, no resistant E. coli were available for MIC90 determination for either drug.

Among the amoxicillin resistance isolates from G1 dogs, no MDR was detected, except for a dog that expressed enrofloxacin resistance. In contrast, all enrofloxacin resistance isolates in G2 expressed MDR (Table 4-4 and Table 4.5).
Table 4-1 Antimicrobial drugs, drug classes and concentrations (µg/ml) determined on Vitek® Gram Negative Veterinary Susceptibility Test Cards (GNS-207) and MIC<sub>BP</sub> (µg/ml) for resistance of each drug based on CLSI guideline (M31-S1)<sup>26-27</sup>

<table>
<thead>
<tr>
<th>Drug classes</th>
<th>Antimicrobial drugs</th>
<th>Concentrations tested</th>
<th>MIC&lt;sub&gt;BP&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Ampicillin (A)</td>
<td>0.5, 4, 32</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin/Clavulanic acid (X)</td>
<td>4/2, 8/4, 18/8</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Carbenicillin (B)</td>
<td>32, 256</td>
<td>≥ 512</td>
</tr>
<tr>
<td></td>
<td>Piperacillin (P)</td>
<td>8, 32, 64</td>
<td>≥ 256</td>
</tr>
<tr>
<td></td>
<td>Ticarcillin (R)</td>
<td>32, 64, 128</td>
<td>≥ 256</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Ceftazidime (Z)</td>
<td>4, 8, 64</td>
<td>≥ 32</td>
</tr>
<tr>
<td></td>
<td>Ceftiofur (C)</td>
<td>2, 4, 8</td>
<td>≥ 8</td>
</tr>
<tr>
<td></td>
<td>Cephalothin (L)</td>
<td>4, 16</td>
<td>≥ 32</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin (F)</td>
<td>1, 4</td>
<td>≥ 4</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacin (E)</td>
<td>0.25, 0.5, 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Amikacin (K)</td>
<td>2, 8, 32</td>
<td>≥ 64</td>
</tr>
<tr>
<td></td>
<td>Gentamicin (G)</td>
<td>0.5, 2, 8</td>
<td>≥ 16</td>
</tr>
<tr>
<td></td>
<td>Tobramycin (M)</td>
<td>0.5, 2, 8</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline (T)</td>
<td>2, 8, 32</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Phenics</td>
<td>Chloramphenicol (H)</td>
<td>1, 8</td>
<td>≥ 32</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Trimethoprim/Sulfamethoxazole (S)</td>
<td>2/38, 8/152</td>
<td>≥ 4/76</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Nitrofurantoin (N)</td>
<td>32</td>
<td>≥ 128</td>
</tr>
</tbody>
</table>
Figure 4-1 Total coliform counts (TCC; ■), total *E. coli* count (TEC; □), and % *E. coli* (%EC; ○–○) at 3 different time points; baseline (B), time at resistance (T), and time at resolution (E) in dogs treated with amoxicillin (G1; A), enrofloxacin (G2; B), and control (G3; C) at comparable time-points. (n=8 except for TEC in T and B in G2; n=4)
Table 4-2 Percent resistant *E. coli* (%REC) to amoxicillin and enrofloxacin from dogs treated with either amoxicillin (G1) or enrofloxacin (G2) at each time-point

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% REC to amoxicillin</th>
<th>% REC to enrofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>T</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0</td>
<td>92.71±2.97</td>
</tr>
<tr>
<td>Enrofloxacinb</td>
<td>0</td>
<td>88.17±12.81</td>
</tr>
<tr>
<td>None(Control)d</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Enrofloxacin resistance exhibited in only 1 dog treated with amoxicillin (G1)

b In enrofloxacin treated dogs (G2), *E. coli* in 4 dogs were undetectable after day 9 of therapy
c Enrofloxacin resistance did not resolve in 1 dog
d No antimicrobial resistance was detected in control dogs

Figure 4-2 Total coliform counts (TCC; ), total *E. coli* count (TEC; ), and % *E. coli* (%EC; —o—) from each dog treated with enrofloxacin at time of resistance (T; D9)
Table 4-3 Etest MIC90 (µg/ml) of antimicrobial resistant fecal *E. coli* to amoxicillin and enrofloxacin from dogs treated with either amoxicillin (G1) or enrofloxacin (G2) at the time at resistance (T)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dogs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC90 (µg/ml)</th>
<th>Amoxicillin</th>
<th>Enrofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>≥ 256</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td>≥ 256</td>
<td>≥ 32</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td>≥ 256</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td></td>
<td>≥ 256</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td></td>
<td>≥ 256</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td></td>
<td>≥ 256</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td></td>
<td>≥ 256</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td></td>
<td>≥ 256</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td></td>
<td>≥ 256</td>
<td>≥ 32</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>≥ 256</td>
<td>≥ 32</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td></td>
<td>≥ 256</td>
<td>≥ 32</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td></td>
<td>≥ 256</td>
<td>≥ 32</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> In enrofloxacin treated dogs (G2), *E. coli* in 4 dogs were undetectable after day 9 of therapy.
Table 4-4 Phenotypes of antimicrobial resistant fecal *E. coli* based on Vitek® Gram Negative Veterinary Susceptibility Test Cards from dogs treated with either amoxicillin (G1; n = 8 dogs) or enrofloxacin (G2; n = 4 dogs) at the time at resistance (T)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of resistance (phenotype)(^a)</th>
<th>Frequency</th>
<th>MDR(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>ABPRLC (2)</td>
<td>32.5%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>ABPRLT (3)</td>
<td>25%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>ABPRLCT (2)</td>
<td>25%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>ABPRLCN (1)</td>
<td>0.83%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>ABPRLFET (1)</td>
<td>2.5%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>ABPRLFETH (1)</td>
<td>12.5%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>ABPRLFETN (1)</td>
<td>1.67%</td>
<td>Y</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>ABPRLFET (2)</td>
<td>15%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>ABPRLFETH (4)</td>
<td>60%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>ABPRXLFETGSH (2)</td>
<td>25%</td>
<td>Y</td>
</tr>
</tbody>
</table>

\(^a\) A = ampicillin; B = Carbenicillin; P = piperacillin; R = Ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/Sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin. Number in parentheses refers to the number of dogs carrying that phenotype.

\(^b\) MDR is defined as resistance to 3 or more unrelated drug classes (see Table 4-1)
Table 4-5 Summarize of level and type of resistance of the antimicrobial resistant fecal *E. coli* used in this study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dog</th>
<th>Time</th>
<th>Phenotypes</th>
<th>Frequency</th>
<th>MDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>A1</td>
<td>T</td>
<td>ABPRLT</td>
<td>100%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td></td>
<td>ABPRLFET</td>
<td>15%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETN</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETH</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td></td>
<td>ABPRLFETH</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>T</td>
<td>ABPRLT</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>T</td>
<td>ABPRLT</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>T</td>
<td>ABPRLCT</td>
<td>100%</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLCT</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A6</td>
<td>T</td>
<td>ABPRLCT</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A7</td>
<td>T</td>
<td>ABPRLC</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLCN</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLC</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A8</td>
<td>T</td>
<td>ABPRLC</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLC</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>E1</td>
<td>T</td>
<td>ABPRLFET</td>
<td>40%</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETH</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRXLFETGSH</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>T</td>
<td>ABPRXLFETGSH</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETH</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>T</td>
<td>ABPRLFETH</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETH</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>T</td>
<td>ABPRLFET</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABPRLFETH</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

*a* A = ampicillin; B = Carbenicillin; P = piperacillin; R = Ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/Sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin

*b* MDR is defined as resistance to 3 or more unrelated drug classes (see Table 4-1)
4.4 Discussion

4.4.1 Total counts

In this study, we demonstrated that amoxicillin therapy had no direct effects on TCC, TEC and %EC, although TCC and TEC significantly differed at T time-point. This could be due to the high variations in numbers of fecal TCC and TEC from triplicate plates per each sampling time. In addition, amoxicillin is a semi-synthetic drug to which microbes may naturally be exposed and develop resistance against it.\(^\text{35}\) However, TEC in this study group was in the same range as that reported in normal fecal *E. coli* in dogs; \(\log_{10}\) of 5.17-10.34 versus 7.5 cells per g feces reported by Sørum and Sunde (2001).\(^\text{11}\)

Enrofloxacin therapy, although had no statistical impact on TCC and TEC, it significantly decreased %EC at resistance time-point. This was probably due to the decrease of TEC after enrofloxacin therapy which may explain the disappearance (undetectable) of *E. coli* in 4 out of 8 dogs after day 9 of enrofloxacin therapy. This phenomenon is supported by the studies of Reeves (1986) and Edlund and Nord (2000) in which FQs had profound impact on aerobic population of the gastrointestinal flora by transiently suppressing or eliminating them. Microbes returned to pre-antimicrobial administration numbers within 2 weeks after cessation of the therapy.\(^\text{9,31}\) Trott *et al* (2004) has also demonstrated similar results in dogs in which animals given a daily oral enrofloxacin at 5 mg/kg for 21 consecutive days exhibited a significant decline in fecal coliforms to undetectable levels by 3 days of therapy and remained suppressed throughout the period of enrofloxacin dosing. Upon termination of antibiotic administration, fecal coliforms gradually returned to levels comparable to those seen prior
to antibiotic treatment by 8 days. In our study, however, disappearance of *E. coli* in those 4 dogs did not return to be detectable post-antimicrobial. This could be due to the total eradication of *E. coli* by enrofloxacin treatment. Another possibility is that enrofloxacin changed the property of *E. coli* isolates such that they were not detected by CHROMagar *E. coli* medium. Although *E. coli* detection by this medium is generally based on the detection of β-glucuronidase (GUD) activity of *E. coli*, which reacts with X-glucuronide and methyl-glucuronide in the medium, the specificity of detection of *E. coli* by CHROMagar *E. coli* medium is about 95-97%. In addition, the prevalence of having positive GUD activity in *E. coli* is about 94-97% in general. Therefore, it is still possible that false negative occurred due to the change of properties of *E. coli* after enrofloxacin treatment.

### 4.4.2 Time periods and percents resistance

In this study, amoxicillin resistance developed rapidly in all dogs within 4-9 days of amoxicillin therapy, and resolved within 6-11 days upon drug-discontinuation. Seven out of 8 amoxicillin-treated dogs (87.5%) exhibited only amoxicillin, but not enrofloxacin, resistance. In the other dog, however, enrofloxacin resistance occurred by day 9 of amoxicillin treatment, with %REC of 0.66, and resolved by 1 week after drug was discontinued. This small percentage of enrofloxacin resistance was less likely to be due to amoxicillin therapy, but may be due to the mechanical transfer of enrofloxacin resistant genes. In amoxicillin-treated dogs, resistance to both amoxicillin and enrofloxacin developed and resolved rapidly upon amoxicillin administration and

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discontinuation, suggesting the horizontal mechanism of resistance gene transfer, such as plasmids. However, this hypothesis needs to be further investigated.

In enrofloxacin-treated dogs, on the other hand, both enrofloxacin and amoxicillin resistance rapidly developed at day 9 of enrofloxacin therapy with resistance resolution varied from 6-27 days for enrofloxacin and 6-13 days for amoxicillin. These findings were different from our pilot data (see chapter 3), in which resistance to both enrofloxacin and amoxicillin developed by day 3 of enrofloxacin treatment; and both enrofloxacin and amoxicillin resistance persisted for at least 3 weeks post-therapy. The more rapid resolution of amoxicillin and enrofloxacin resistance in this study could be from more restricted environment and husbandry procedures to limit the contact between dogs and minimize the transfer of resistance genes among dogs.

4.4.3 Level and type of antimicrobial resistance

Amoxicillin resistance mediated non-MDR in fecal E. coli in this study. This could be due to the predominant mechanism of amoxicillin resistance in Gram-negative bacteria, especially in intestinal coliforms and in E. coli, the production of β-lactamases. Beta-lactamases are the specific enzymes which are encoded either chromosomally or by plasmids and inactivate β-lactams by hydrolyzing the four-membered β-lactam rings. However, resistance to tetracycline, but not other drugs, was noticed in approximately 50% of these non-MDR isolates, indicating the involvement of either a resistance mechanism which is more specific to the tetracycline resistance genes rather than other
non-specific mechanisms such as efflux system and permeability through porin, or the horizontal transfer of tetracycline resistance genes among dogs.\textsuperscript{28, 37-39}

Enrofloxacin, but not amoxicillin therapy, mediated MDR, with \textit{E. coli} resistant to \(\beta\)-lactam drugs being particularly present in this study. FQ resistant \textit{E. coli} has been reported to exhibit resistance to other drugs such as ampicillin, tetracycline, chloramphenicol, gentamicin, and trimethoprim/Sulfamethoxazole which are all similar to our findings in this study.\textsuperscript{40-41} These findings suggest different mechanisms of resistance between amoxicillin and enrofloxacin. Enrofloxacin is a veterinary FQ whose resistance is mainly due to mutations in bacterial \textit{gyrA} and \textit{parC} genes that code for DNA gyrase and topoisomerase IV enzymes.\textsuperscript{43-45} However, decreased concentration of FQ in bacterial cell via efflux pumps, for example AcrAB-TolC efflux system, plays an important role for FQ-mediated MDR in \textit{E. coli}.\textsuperscript{46} This efflux system mediates not only resistance to FQ, but also to other drugs such as ampicillin, tetracycline, chloramphenicol, rifampicin, puromycin, organic solvents, pine oils, dyes, disinfectants and detergents.\textsuperscript{44-47} Recently, horizontal transfer of resistance genes by either plasmids or other mobile DNA, such as transposons which involves the transfer of FQ resistant efflux pump genes, has been reported as another mechanism of FQ resistance.\textsuperscript{48-52} This mechanism provides the genetic linkage between resistance to FQ and \(\beta\)-lactam drugs.\textsuperscript{49} Moreover, plasmid transfer of FQ resistance genes may explain the rapid onset of FQ resistance, as well as high-level of FQ resistance in addition to mutations in bacterial DNA gyrase and/or topoisomerase IV enzymes.\textsuperscript{48}
4.5 Conclusion

In this study, either amoxicillin or enrofloxacin at recommended dosing regimens was associated with rapid emergence of high-level resistance to the treatment drug in fecal *E. coli* in dogs. Differences in levels of resistance, MDR for enrofloxacin versus non-MDR for amoxicillin, may reflect the source and mechanism of resistance of drug.

This study suggests that use of all antimicrobials should be judicious, not only to pathogenic but also commensal bacteria. Extra caution is indicated for FQ so that emergence of MDR *E. coli* may be avoided. Genotypic characterization and mechanisms of resistance in non-MDR versus MDR isolates need to be further studied to explain, more in depth, the impact of antimicrobial therapy on the advent of antimicrobial resistance and the mechanism of resistance genes transfer in commensal *E. coli* in dogs.

4.6 References

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37. Levy SB. Tetracycline resistance determinants are widespread. ASM news 54; 418-421.


CHAPTER 5

GENOTYPIC VARIATION AMONG ANTIMICROBIAL THERAPY-INDUCED MDR AND NON-MDR FECAL Escherichia coli IN DOGS

5.1 Introduction

Emergence of multidrug resistant (MDR) E. coli in response to routine antimicrobial therapy is increasingly problematic, not just in human but also in veterinary medicine. The presence and spread of MDR in pathogenic bacteria have decreased the treatment options to a narrow range of drugs that are difficult to administer, potentially toxic, and expensive. In the last two decades, MDR have spread widely among Gram negative bacteria, especially E. coli.

From our previous study (see chapter 4), fluoroquinolones (FQ) tends to induce the emergence of MDR, whereas amoxicillin tends to induce non-MDR in fecal E. coli. Enrofloxacin-associated MDR is accompanied by resistance to amoxicillin. In contrast, resistance to enrofloxacin does not occur in amoxicillin-treated animals. In general, amoxicillin resistance is primarily mediated by β-lactamases, which hydrolyse the β-lactam ring. Resistance to β-lactams tends to be transmitted via plasmids. In contrast, enrofloxacin resistance is mainly mediated by point mutations on genes coding for
expression of the target proteins. These mutations occur primarily in highly conserved regions or quinolone-resistance-determining region (QRDR) of bacterial DNA gyrase and topoisomerase IV enzymes.\textsuperscript{8-15} However, because point mutations are not likely to impact non-fluoroquinolone drugs, expression of MDR must involve other mechanisms.

The purpose of this study was to characterize the presence of extended-spectrum beta-lactamases (ESBL), and genotypic expression using pulse-field gel electrophoresis (PFGE), in non-MDR and MDR fecal \textit{E. coli} isolates from dogs receiving treatment with either amoxicillin or enrofloxacin at recommended dosing regimens.

5.2 Materials and Methods

5.2.1 MDR and non-MDR \textit{E. coli} isolates

A total of 180 MDR and non-MDR fecal \textit{E. coli} isolates were used in this study. All isolates were received from our previous study by the following methods.

Twenty-four healthy, antimicrobial-free, purpose-bred adult hound dogs were studied. All dogs were maintained with regular adult maintenance diet (Hill’s® Science Diet® Adult Large Breed dry food, Hill’s Pet Nutrition Inc., Topeka, KS). Each dog was randomly housed in individual, climate controlled cage in a strictly-controlled environment, as well as kennel access limitation to minimize mechanical transmission of microbes and antimicrobial resistance genes. Dogs were randomly divided in three groups of eight. Each group received a different antimicrobial therapy: group1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 h, group2 (G2) was treated with 5
mg/kg enrofloxacin orally every 24 h, and Group 3 (G3) received no treatment and was reserved as control group. Both drugs were administered for 7 to 21 days, with the duration based on the emergence of antimicrobial resistance, defined as a proportion of total resistant *E. coli* to total *E. coli* counts ≥75%. The time at ≥75% resistance was defined as time of resistance (T), and then antimicrobial therapy was discontinued.

Fresh fecal samples were obtained by digitally collected *per rectum* into sterile containers prior to (Baseline; B) and every 3 days during treatment until time of resistance (T). Drug was then discontinued and monitoring continued weekly up to 4 weeks, or until the resistance resolved (≤25% of cfu resistant or study end; E), whichever came first. If the resistance did not resolve by 4 weeks post-therapy, E would be reported as 4 weeks.

Antimicrobial resistant *E. coli* were obtained randomly from the 18-24 h growth of fecal dilutions (with 0.9% saline solution) at 37°C on CHROMagar® *E. coli* agar plates (CHROMagar, Paris, France) containing either amoxicillin or enrofloxacin at concentrations 1 tube dilution below the breakpoint MIC (MIC_{BP}) of either drug: at 16 µg/ml agar for amoxicillin and 2 µg/ml agar for enrofloxacin respectively.

From each of the three time points (B, T or E) for each group of dogs, 10 *E. coli* colonies were randomly selected from the population of antimicrobial resistant *E. coli* recovered from dogs receiving either amoxicillin (n=8 dogs) or enrofloxacin (n=4 dogs), with a total of 180 isolates. Susceptibility to amoxicillin and enrofloxacin was performed using Etest® (Epsilometer or Epsilon; AB Biodisk/ BioMérieux, Inc. Hazelwood, MO) strips and phenotype of each isolates was determined using Vitek® automated system.
with Gram Negative Veterinary Susceptibility Test Cards (GNS-207) based on the susceptibility to 8 antibacterial classes; 17 antimicrobial drugs including Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Carbenicillin, Ceftazidime, Ceftiofur, Cephalothin, Chloramphenicol, Ciprofloxacin, Enrofloxacin, Gentamicin, Nitrofurantoin, Piperacillin, Tetracycline, Ticarcillin, Tobramycin, and Trimethoprim/Sulfamethoxazole. Presence of multidrug resistance (MDR) was indicated if \( E. \ coli \) was resistant to 3 or more unrelated drug classes. Summarize of phenotypes and types of resistance (non-MDR vs. MDR) of the isolates are shown in Table 5-1.

5.2.2 Genotypes

Genomic fingerprints were determined in all 180 antimicrobial resistant isolates using Pulse-field gel electrophoresis (PFGE) in accordance with the Pulse-Net standardized protocol for molecular subtyping of \( E. \ coli \ O157:H7 \).\(^{16}\) Briefly, antimicrobial resistant isolates were grown for 14 to 18 hours before being suspended cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0). Concentrations (turbidity) of cell suspensions were adjusted to the absorbance of 1.3-1.4 at 610 nm wavelength in a spectrophotometer. Plugs were prepared by mixing 400 µl of cell suspension, 20 µl of a 2% proteinase K solution, and an equal volume of 1% SeaKem Gold:1% sodium dodecyl sulfate (SDS) agarose and dispensed into reusable plug molds. Plugs were allowed to solidify at room temperature for 10-15 minutes before removed from the molds. Cells embedded in plugs were lysed in cell lysis buffer containing 20 mg/ml proteinase K in a shaker water bath at 54°C, 150-175 rpm for 1.5-2 h. Lysed plugs were washed with
preheated sterile ultrapure water and TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) respectively. Plugs were then digested with a restriction enzyme XbaI (50 U/plug) and incubated at 37°C for 1.5-2 h. The resolution of the generated DNA fragments was obtained with 1% SeaKem Gold Agarose gels in 0.5×Tris-Borate EDTA Buffer at 14°C; initial switch time 5 s; final switch time 40 s; duration of run 18 h, angle, 120°; gradient, 6 V/cm with a linear ramping factor using a CHEF MAPPER II System (Bio-Rad, Hercules, CA). Pattern images were acquired by staining gels with ethidium bromide at a final concentration of 10 µg/ml. Salmonella enterica serovar Braenderup H9812 was used as the size standard strain.

Tiff images of the PFGE gels were analysed using the BioNumerics® software, version 4.5 (Applied Maths, Austin, TX). Analysis of band patterns and construction of dendrograms were performed using the Dice correlation coefficient and clustering of patterns was performed by unweighted pair group with arithmetic averaging (UPGMA). Ninety percent of similarity between patterns was used to address the genetic relatedness among the PFGE patterns.17
Table 5-1 Summary of the type and proportion of antimicrobial resistance and the PFGE-based genotypes of the MDR and non-MDR fecal *E. coli* isolates used in this study (n=180)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dog</th>
<th>Time</th>
<th>Phenotypes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion</th>
<th>MDR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>A1</td>
<td>T</td>
<td>A BPRL T</td>
<td>100%</td>
<td>N</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>T</td>
<td>A BPRL T</td>
<td>100%</td>
<td>N</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>T</td>
<td>A BPRL T</td>
<td>100%</td>
<td>N</td>
<td>4, 5, 6, 7</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>T</td>
<td>A BPRL C T</td>
<td>100%</td>
<td>N</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>A BPRL C T</td>
<td>100%</td>
<td>N</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>T</td>
<td>A BPRL C T</td>
<td>100%</td>
<td>N</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>A BPRL C N</td>
<td>100%</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>A6</td>
<td>T</td>
<td>A BPRL C A BPRL C N</td>
<td>90%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>A BPRL C</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A7</td>
<td>T</td>
<td>A BPRL C</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>A BPRL C</td>
<td>100%</td>
<td></td>
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<tr>
<td></td>
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<td>E</td>
<td>A BPRL FE T H</td>
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<tr>
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<tr>
<td></td>
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<sup>a</sup> A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin

<sup>b</sup> MDR is defined as resistance to 3 or more unrelated drug classes (see Table 4-1). T refers to the time period that the sample was collected.
5.2.3 Presence of extended-spectrum beta-lactamases (ESBLs)

Three representative isolates from each genotype (based on PFGE dendrogram) within each phenotype were randomly selected for ESBLs determination using microdilution-based Sensititre® (TREK diagnostic systems, Cleveland, OH) with ESBL Confirmatory MIC plates (ESB1F). Presence of ESBL was based on susceptibility to 9 single β-lactam drugs; Ampicillin, Cefazolin, Cephalothin, Cefoxitin, Cefipime, Cefpodoxime, Ceftriaxone, Cefotaxime, Ceftazidime, 3 extended(broad)-spectrum β-lactam drugs; Cefotaxime/Clavulanic acid, Ceftazidime/Clavulanic acid, Piperacillin/Tazobactam, 2 carbapenems; Meropenem, Imipenem, and 2 other drugs; Ciprofloxacin, and Gentamicin (Table 5-2) according to the manufacturer’s instructions. Briefly, each 18 to 24 h growth isolate was emulsified in 4 ml of 0.9% sodium chloride solution and adjusted to a McFarland turbidity standard of 0.5 using the Sensititre nephelometer (TREK diagnostic systems, Cleveland, OH). For each cell suspension, 10 μl was transferred to 11 ml of cation-adjusted Mueller-Hinton broth and mixed. Then 50 μl of the broth suspension was transferred to each well of ESBL Confirmatory MIC plates by Sensititre® Autoinoculator. Plates were covered and sealed with the adhesive seals before being incubated at 35°C. After 18-24 h incubation at 35°C, all plates were read using the Vizion™ System with SWIN™ software. An image of the growth in each well from Vizion™ System was displayed on a touch screen monitor with the plate's antimicrobial template overlaid on the image. MICs were interpreted based on the growth in each well.
The susceptibility status of each drug was based on comparisons of the MIC for that drug to CLSI antimicrobial susceptibility standards as delineated in M100-S16.\(^\text{19}\)

ESBL was confirmed if a decreased MIC $\geq 3$ two-fold dilution for $\beta$-lactam tested in combination with clavulanic acid or tazobactam versus its MIC when tested alone.

Classifications of lactamase enzymes are shown in Table 5-3.

Table 5-2 Antimicrobial drugs, drug classes and dilution range (µg/ml) determined on Sensititre Vizion System® ESBL Confirmatory MIC plates (ESB1F) and MIC\(_{\text{BP}}\) (µg/ml) for resistance of each drug based on CLSI guideline (M100-S16)\(^\text{19}\). The generation for each cephalosporin is indicated.

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<tr>
<th>Drug groups</th>
<th>Abbreviations</th>
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<th>Dilution range</th>
<th>MIC(_{\text{BP}})</th>
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<td>T/C</td>
<td>Ceftazidime/Clavulanic acid</td>
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<td>P/T</td>
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<td>Gentamicin</td>
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Table 5-3 Classification schemes of β-lactamase enzymes

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<tr>
<th>Classifications</th>
<th>Bush-Jacoby-Medeiros</th>
<th>Molecular (amber) class</th>
<th>β-lactam drugs (substrates)</th>
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<td>A</td>
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<td>Penicillins</td>
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<td>D</td>
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5.3 Results

5.3.1 PFGE Genotypes

Most of the isolates recovered from amoxicillin-treated dogs were non-MDR, except for those from 1 dog that exhibited MDR. Among 4 phenotypes of these non-

128
MDR isolates, total of 10 PEGE patterns (with ≥90% homology) were identified, with various genotypes within each phenotype (Figure 5-1), except for those that were susceptible to tetracycline (ABPRLC and ABPRLCN) for which 1 genotype was present (Table 5-1, Figure 5-2). For those isolates in the one amoxicillin-treated dog that exhibited MDR, (3 phenotypes) only 1 genotype was characterized from 3 phenotypes (Figures 5-3 and 5-4).

All the isolates recovered from enrofloxacin-treated dogs were MDR. Among 3 phenotypes, 2 PFGE patterns emerged (Figure 5-5). However, those genotypes were distinct from a pattern exhibited in MDR isolates recovered from an amoxicillin-treated dog (Figures 5-3, 5-5 and 5-6).

5.3.2 Presence of ESBLs

All non-MDR and MDR isolates were susceptible to ceftazidime and carbapenems (imipenem and meropenem) but resistant to ampicillin (Tables 5-4 and 5-5). However, susceptibility to the remaining cephalosporins varied.

Resistance to ceftiofur was detected only in Non-MDR isolates and was associated with ESBL; with ≥3 two-fold dilution decreases in MICs of the third generation cephalosporins in the presence of clavulanic acid or tazobactam, compared to MICs of the single drugs. In contrast, no ESBL was found in non-MDR isolates that were susceptible to ceftiofur. All non-MDR isolates were also susceptible to first (except cephalothin in some phenotypes), second and fourth generation cephalosporins as well as
ciprofloxacin and gentamicin, confirming the phenotypes indicated by Vitek® automated susceptibility testing. Non-MDR isolates that were resistant to ceftiofur were also resistant to first, third (except ceftazidime) and fourth generation cephalosporins. However, resistance to second generation cephalosporins varied among non-MDR isolates.

The presence of ESBL in MDR isolates varied with the treatment drug. For enrofloxacin-treated dogs, no resistance was exhibited toward the 3rd generation drug ceftazidime, 4th generation cefipime or the carbapenems. However, MDR isolates were resistant to ampicillin, the 1st and 2nd generation cephalosporins, to the 3rd generation drug cefpodoxime (but susceptible to ceftiofur), and non-beta-lactams ciprofloxacin +/- gentamicin. No ESBL was detected in any MDR isolates.

MDR isolates from a dog treated with amoxicillin were susceptible to third and fourth generation cephalosporins, and gentamicin in addition to carbapenems but resistance to ciprofloxacin and ampicillin. Moreover, susceptibility to first and second generation cephalosporins of these MDR isolates varied.
Figure 5-1 Representative PFGE gel of amoxicillin-mediated non-MDR *E. coli* isolates from 1 phenotype ABPRLT (lanes 2-7, 9-14). Lanes 1, 8, 15 represent *Salmonella enterica* serovar Braenderup H9812 as the size standard strain.

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; T = tetracycline)
Figure 5-2 Representative PFGE gel of amoxicillin-mediated non-MDR *E. coli* isolates from 2 phenotype ABPRL (lanes 2-3, 5-7, 9-14) and ABPRLCN (lane 4). Lanes 1, 8, 15 represent *Salmonella enterica* serovar Braenderup H9812 as the size standard strain.

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; T = tetracycline; N = nitrofurantoin)
Figure 5-3 Representative PFGE gel of MDR *E. coli* isolates associated with amoxicillin treatment from 3 phenotype ABPRLFET (lanes 2, 4, 6), ABPRLFETH (lanes 5, 9-14) and ABPRLFETN (lanes 3, 7). Lanes 1, 8, 15 represent *Salmonella enterica* serovar Braenderup H9812 as the size standard strain.

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin)
Figure 5-4 Representative dendrograms of non-MDR isolates

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin)
Figure 5-5 Representative PFGE gel of enrofloxacin-mediated MDR *E. coli* isolates associated with from 3 phenotype ABPRLFET (lanes 2-3, 8-10), ABPRLFETH (lanes 4-6) and ABPRXLFTGSH (lanes 12-14). Lanes 1, 7, 15 represent *Salmonella enterica* serovar Braenderup H9812 as the size standard strain.

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin)
Figure 5-6 Representative dendrogram of MDR isolates from enrofloxacin treated dogs

(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin)
Table 5-4 MICs and the presence of ESBL in representative MDR and non-MDR isolates, 3 isolates from each genotype within phenotype

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*only 1 isolate present in this category

**only 2 isolates present in these categories

(Gen = genotypes, ID = isolate id, AMP = ampicillin, FAZ = cefazolin, CEP = cephalothin, FOX = cefoxitin, FEP = cefipime, POD = cefpodoxime, AXO = ceftriaxone, FOT = cefotaxime, TAZ = ceftazidime, F/C = cefotaxime/clavulanic acid, T/C = ceftazidime/clavulanic acid, P/T = piperacillin/tazobactam, IMI = imipenem, MER = meropenem, CIP = ciprofloxacin, GEN = gentamicin)
Table 5-5 Presence of ESBL in representative MDR and non-MDR isolates

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<td></td>
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<td>A BPRLC N</td>
<td></td>
<td>1</td>
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<td></td>
<td>AXBPRL FE T H G S</td>
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(A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin; Y = yes; N = no)

5.4 Discussion

In this study, information regarding post-treatment resistance was not available for control animals for which no resistance was present despite monitoring at the same time points as treated dogs. No antimicrobial resistant *E. coli* were detected in any samples from control dogs at any time-point.

In amoxicillin treated dogs, apart from 1 dog that exhibited MDR, all isolates were non-MDR. Among 4 phenotypes of those non-MDR isolates, 10 different PFGE
profiles were obtained, demonstrating that there was not a unique antimicrobial resistant fecal *E. coli* clone mediated by amoxicillin treatment among and within the dogs. In addition, it was notable that in some non-MDR isolates that shared the same phenotypes where characterized by multiple PFGE profiles. These findings suggest the involvement of transferrable (horizontal) mechanisms of β-lactam resistance such as plasmid-mediated β-lactamases. Interestingly, non-MDR phenotypes that conferred resistance to tetracycline were characterized by multiple PFGE patterns compared to phenotypes without tetracycline resistant, for which only 1 profile was detected. This also supports the involvement of plasmid or horizontal mechanisms of antimicrobial resistance, which is also a common mechanism of tetracycline resistance in *E. coli*. For those non-MDR isolates that were susceptible to tetracycline, 1 genotype was manifested as 2 phenotypes, which differed in susceptibility to nitrofurantoin. This could be due to the mechanism of resistance to nitrofurans including nitrofurantoin, which is based on the stepwise mutation that may not be differentiated from the wild type by PFGE.

Interestingly, only one PFGE pattern was exhibited among 3 distinct MDR phenotypes obtained from a dog treated with amoxicillin, suggesting a unique clone of MDR *E. coli*. This clone is distinct from the other 10 profiles recovered from non-MDR isolates treated with the same drug, amoxicillin. How enrofloxacin resistance occurred in amoxicillin-treated dog, although at a low prevalence, is not well explained. It is possible that, despite attention to husbandry and hygiene, FQ resistant genes on plasmids or other transferrable DNA were transferred by fomites from enrofloxacin-treated dogs. Examples might be efflux pump genes, which is more likely from this study, although it is less common in nature.
In MDR isolates from enrofloxacin treated dogs, only 2 PFGE profiles yielded 3 distinct phenotypes among the 4 dogs. Among these profiles, none shared the same MDR pattern as occurred in the amoxicillin-treated dog. This demonstrates that MDR isolates from enrofloxacin therapy are not genetically related to those MDR obtained from the amoxicillin-treated dog and suggests a different mechanism by which the resistance emerged. More importantly, mechanisms of MDR mediated by enrofloxacin therapy are more unique among phenotypes and different from those of non-MDR. This hypothesis is different from that reported in clinical *E. coli* isolates in which FQ resistant isolates were genetically diverse, not clonal.\(^ {32-33}\) This could be because resistance to enrofloxacin in this study was obtained from feces of healthy dogs that were treated with enrofloxacin at the similar regimen and housed in the same kennel with similar food and other environmental conditions. However, FQ resistance reported in clinical *E. coli* isolates was mostly from diseased humans or dogs in with varied background such as treatment and environment that may impact the varieties of the clones.

Resistance to enrofloxacin and amoxicillin in *E. coli* is usually caused by unlinked mechanisms. While β-lactam resistance is mainly caused by plasmid-mediated mechanisms such as β-lactamases, resistance to FQ is mostly mediated by mutations in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC* genes.\(^ {12-15}\) Cross-resistance to other drugs has been frequently reported in combination with fluoroquinolone resistance, especially with high-level FQ resistance.\(^ {34-39}\) Mechanisms responsible for MDR in *E. coli* vary from the involvement of drug efflux pump, whose genes are located on chromosomes, to non-specifically move drugs out of bacterial cells, to the genes located on mobile DNAs such as plasmids and integrons, as well as
transferrable mechanisms that transfer chromosomal genes to other bacterial cells via transferrable DNA.\textsuperscript{30, 34, 37, 39-47}

For the presence of ESBLs, only non-MDR phenotypes with resistance to ceftiofur exhibited ESBLs (Table 5-5). Ceftiofur is a third generation veterinary cephalosporin.\textsuperscript{48} Resistance to this group of cephalosporin in addition to other extended-spectrum penicillins, first, third and forth generation cephalosporins suggests the plasmid-mediated $\beta$-lactamases group 2be based on Bush-Jacoby-Medeiros (amber group A) (Table 5-3). Other non-MDR phenotypes without resistance to ceftiofur, on the other hand, did not exhibit ESBL, but resistance to first generation cephalosporin (cephalothin $\pm$ cefazolin). This suggests another group of $\beta$-lactamases, either 2b or 2c (both share the same amber class A) to be involved in these isolates. The differences between $\beta$-lactamases group 2b and 2c are the resistance to narrow-spectrum cephalosporins such as cefazolin, cephalothin (first generation cephalosporin) in group 2b.\textsuperscript{8, 21, 47-50} Interestingly, all non-MDR isolates were susceptible to clavulanic acid, whereas susceptibility to cephapemycins (cefoxitin; second generation cephalosporin) varied. However, resistance to cephapemycins is not generally due to the presence of $\beta$-lactamases, but may be due to the changes in porins in outer membrane which decrease or eliminate flow of small hydrophilic molecules like $\beta$-lactam drugs or the multidrug transporter such as AcrAB/TolC in \textit{E. coli} which mediates excretion of $\beta$-lactams.\textsuperscript{4, 51}

Neither MDR isolates from amoxicillin nor enrofloxacin treatment exhibited ESBL. On the other hand, they were generally susceptible to third and forth generation cephalosporins, but resistance to first generation generation drugs, indicating less
involvement of ESBL. If β-lactamases were present in these MDR isolates, they tend to be from group 2b (amber class A) based on the resistance pattern, which is similar to that suggested in non-MDR isolates that did not exhibit ESBL. However, resistance to β-lactam drugs in MDR may be mediated by other mechanisms beyond β-lactamases. No isolates were resistance to carbapenems, suggesting the irrelevance to groups 2df, 2f and 3. However, the presence of β-lactamase genes need to be further investigated in details before the involvement of β-lactamases in non-MDR and MDR E. coli mediated by antimicrobial therapy is addressed.

5.5 Conclusion

These data suggest that therapeutic doses of popular antimicrobials are associated with clonal expansion of resistant isolates for the respective drug. However, non-MDR isolates from amoxicillin treatment tend to have variable phenotypes and genotypes, whereas MDR isolates from enrofloxacin treatment tended to be more genotypically similar, regardless of phenotypes. In addition, ESBLs were induced by amoxicillin therapy, but not enrofloxacin. This study suggests that different mechanisms of resistance in fecal E. coli are mediated by amoxicillin and enrofloxacin, potentially supporting the role of plasmids (e.g. beta-lactamases) in amoxicillin resistance, compared to mutation-mediated resistance for enrofloxacin as well as MDR.
5.6 References


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CHAPTER 6

MECHANISMS OF ANTIMICROBIAL-MEDIATED MDR AND NON-MDR *Escherichia coli* IN FECES OF HEALTHY DOGS

6.1 Introduction

Emergence of multidrug resistance (MDR) *E. coli* in response to routine antimicrobial therapy is increasingly problematic in veterinary medicine.\(^1\)\(^-\)\(^6\) From our previous study of fecal *E. coli* exposed to amoxicillin or enrofloxacin, the fluoroquinolone (FQ) induced not only self-resistance, but also resistance to multiple drugs (MDR), whereas amoxicillin induced non-MDR. Enrofloxacin-associated MDR was accompanied by resistance to amoxicillin, however, resistance to enrofloxacin did not occur in amoxicillin-treated dogs.

In general, amoxicillin resistance is most frequently mediated by β-lactamase enzymes, which inactivate β-lactam drugs by hydrolyzing their β-lactam ring. Resistance to β-lactams tends to be horizontally transmitted via transferrable genetic materials such as plasmids.\(^7\)\(^-\)\(^16\) In contrast, enrofloxacin resistance is mainly mediated by point mutations on genes coding for expression of the target proteins. These mutations occur primarily in highly conserved regions or quinolone-resistance-determining region.
(QRDR) of bacterial DNA gyrase and topoisomerase IV enzymes.\textsuperscript{17-20} However, because point mutations in the target protein should be specific to the target drug or drug class (i.e. FQ), expression of MDR induced by enrofloxacin therapy must involve other mechanisms.

The purpose of this study was to characterize the mechanisms by which amoxicillin and enrofloxacin induce antimicrobial resistance (both non-MDR and MDR) in fecal \textit{E. coli}. Using fecal \textit{E. coli} isolates from dogs receiving treatment with either amoxicillin or enrofloxacin at recommended dosing regimens, we investigated the potential roles of β-lactamase enzymes, mutations in QRDRs of \textit{gyrA}, \textit{gyrB} and \textit{parC} genes, MDR pumps and general regulators in both non-MDR and MDR isolates. Further, the potential horizontal transfer of the resistance genes by conjugation was studied.

6.2 Materials and Methods

6.2.1 Fecal \textit{E. coli} isolates

A total of 18 MDR (n=6) and non-MDR (n=11) fecal \textit{E. coli} isolates were studied. Our previous studies had demonstrated multiple genotypes occurred in each phenotype. Within each group, each genotype within each phenotype was represented. All isolates were cultured from our previous studies using the following methods.

Twenty-four healthy, antimicrobial-free, purpose-bred adult hound dogs were studied. All dogs were maintained with regular adult maintenance diet (Hill’s® Science Diet® Adult Large Breed dry food, Hill’s Pet Nutrition Inc., Topeka, KS). Each dog was
randomly housed in individual, climate controlled cage in a strictly-controlled environment, as well as kennel access limitation to minimize mechanical transmission of microbes and antimicrobial resistance genes. Dogs were randomly divided in three groups of eight. Each group received a different antimicrobial therapy: group 1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 h, group 2 (G2) was treated with 5 mg/kg enrofloxacin orally every 24 h, and Group 3 (G3) received no treatment and was reserved as control group. Both drugs were administered for 7 to 21 days, with the duration based on the emergence of antimicrobial resistance, defined as a proportion of total resistant *E. coli* to total *E. coli* counts ≥75%. The time at ≥75% resistance was defined as time of resistance (T), and then antimicrobial therapy was discontinued.

Fresh fecal samples were obtained by digitally collected *per rectum* into sterile containers prior to (Baseline; B) and every 3 days during treatment until time of resistance (T). Drug was then discontinued and monitoring continued weekly up to 4 weeks, or until the resistance resolved (≤25% of cfu resistant or study end; E), whichever came first. If the resistance did not resolve by 4 weeks post-therapy, E would be reported as 4 weeks.

Antimicrobial resistant *E. coli* were obtained randomly from the 18-24 h growth of fecal dilutions (with 0.9% saline solution) at 37°C on CHROMagar® *E. coli* agar plates (CHROMagar, Paris, France) containing either amoxicillin or enrofloxacin at concentrations 1 tube dilution below the breakpoint MIC (MIC<sub>BP</sub>) of either drug: at 16 µg/ml agar for amoxicillin and 2 µg/ml agar for enrofloxacin respectively. Ten
representative antimicrobial susceptible isolates were obtained from fecal samples of control, untreated dogs, at baseline (B; D0).

6.2.2 MDR and non-MDR fecal *E. coli* isolates

From each of the three time points (B, T or E) for each group of dogs, 10 *E. coli* colonies were randomly selected from the population of antimicrobial resistant *E. coli* recovered from dogs receiving either amoxicillin (n=8 dogs) or enrofloxacin (n=4 dogs), from a total of 180 isolates. Levels of resistance (susceptibility) to amoxicillin and enrofloxacin, and type of resistance were performed using Etest® (Epsilometer or Epsilon; AB Biodisk/ BioMérieux, Inc. Hazelwood, MO) strips and Vitek® automated system (BioMérieux, Inc. Hazelwood, MO) with Gram Negative Veterinary Susceptibility Test Cards (GNS-207) based on the susceptibility to 8 antibacterial classes; 17 antimicrobial drugs including Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Carbenicillin, Ceftazidime, Ceftiofur, Cephalothin, Chloramphenicol, Ciprofloxacin, Enrofloxacin, Gentamicin, Nitrofurantoin, Piperacillin, Tetracycline, Ticarcillin, Tobramycin, and Trimethoprim/Sulfamethoxazole, respectively. Multidrug resistance (MDR) was indicated if *E. coli* was resistant to 3 or more unrelated drug classes.

Genomic fingerprints were determined in all 190 antimicrobial resistant isolates using Pulse-field gel electrophoresis (PFGE) in accordance with the Pulse-Net standardized protocol for molecular subtyping of *E. coli* O157:H7. Analysis of band patterns and construction of dendrograms were performed using the Dice correlation
coefficient and clustering of patterns was performed by unweighted pair group with arithmetic averaging (UPGMA) with 90 percent of similarity between patterns to address the genetic relatedness among the PFGE patterns.

6.2.3 Conjugative transfer of resistance genes

In order to determine the transmissibility of the resistance determinants, conjugation was performed on all 18 representative non-MDR and MDR *E. coli* isolates. In this study, conjugation assays were carried out on both liquid and solid surface to obtain and/or confirm the maximum conjugation frequency.

6.2.3.1 Conjugation in broth

Horizontal transfer of antimicrobial resistance genes were investigated in all isolates with conjugation method modified from Miller (1972). Briefly, all isolates were grown on Luria-Bertani (LB) broth to logarithmic growth phase (1-2x10^8 viable cells per ml). Conjugations were performed by mixing equal amount of cultures of each non-MDR or MDR *E. coli* isolate with the recipient GN 3201 *E. coli* strain. This strain contains Tn5, which carries a kanamycin resistance gene. The positive control was CAG 5051 Hfr *E. coli* with Tn10 that includes a tetracycline resistance gene. Mating occurred for 1 h at 37°C. Serial 10-fold dilutions of the culture were then made in 0.9% saline solution. Transconjugants were selected by plating the appropriate dilutions on LB agar plates containing kanamycin, kanamycin + amoxicillin, kanamycin + enrofloxacin, and
enrofloxacin at the drug concentration one tube below the MICBP (50 µg/ml kanamycin, 16 µg/ml amoxicillin and 2 µg/ml enrofloxacin, respectively). Positive control was selected on LB plates containing tetracycline at the concentration one tube below the MICBP (8 µg/ml). All the plates were incubated at 37°C for 24-48 h.

6.2.3.2 Conjugation on solid surface

Conjugation on a solid surface was also performed in all isolates, to confirm the results from conjugation in liquid media, by the method modified from Miller (1972).21 Briefly, all isolates were grown on Luria-Bertani (LB) broth to logarithmic growth phase (1-2x10⁸ viable cells per ml). Conjugations were performed by mixing equal amount of cultures of each non-MDR or MDR E. coli isolates and one of 3 different recipients; GN 3201 E. coli strain containing Tn5 (Kan'), DH5α E. coli strain (recA⁻, Nal') or TB1 Hfr E. coli strain with (recA⁺, Nal'). TB1 Hfr E. coli strain with (recA⁺, Nal') were used as a recipient with isolates that are resistant to kanamycin (cannot be tested with GN 3201 E. coli strain). CAG 5051 Hfr E. coli containing Tn10 with tetracycline-resistant mutant was used as a positive control. The suspensions were spotted on LB plates. The mating was allowed to continued on LB plates overnight at 37°C. Cells were randomly picked up with the sterile cotton swab and resuspended in 0.9% Saline solution. Serial 10-fold dilutions of the culture were further made in 0.9% Saline solution. Transconjugants were selected by plating the appropriate dilutions on LB agar plates containing nalidixic acid, nalidixic acid + amoxicillin, nalidixic acid + enrofloxacin, and enrofloxacin at the drug concentration one tube below the MICBP (50 µg/ml kanamycin, 16 µg/ml amoxicillin and
2 µg/ml enrofloxacin, respectively). Positive control was selected on LB plates containing tetracycline at the concentration one tube below the MIC_{BP} (8 µg/ml). All the plates were incubated at 37°C for 24-48 h.

6.2.3 Detection of presence and mutations of resistance genes

PCR amplification of the QRDRs of DNA gyrase (\textit{gyrA} and \textit{gyrB}), topoisomerase IV (\textit{parC}), β-lactamase (TEM, SHV and PSE-1), AcrAB-TolC efflux pump (\textit{acrR}) and general gene regulator (\textit{soxS}) were performed to detect and identify mutations in all 18 representative isolates. \textit{W3110} \textit{E. coli} strain (wild-type) was used as a control isolates. With exception of \textit{gyrA}, \textit{parC} and \textit{gyrB} primers, all oligonucleotide primers were designed using Lasergene software package (DNASTAR, Madison, MI). DNA sequencing was performed in DNA gyrase (\textit{gyrA} and \textit{gyrB}) and topoisomerase IV (\textit{parC}) genes. The oligonucleotide primers used for PCR amplification and DNA sequencing are described in Table 6-1.

A single colony of each bacterial isolate was used as the template for PCR amplification. The PCR was performed using \textit{Taq} DNA polymerase (New England Biolabs, Ipswich, MA) in a Multigene\textsuperscript{TM} Gradient Thermocycler PCR System (Labnet International Inc, Edison, NJ.). The PCR products were purified using a Qiaquick PCR purification kits (Qiagen, Inc., Valencia, CA). DNA sequencing was performed at the DNA Core Facility, Massachusetts General Hospital (Cambridge, MA).
### Table 6-1 Oligonucleotides used for PCR and DNA sequencing

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### 6.3 Results

#### 6.3.1 Conjugative transfer of resistance genes

Bacterial conjugation, both plate and broth methods, revealed high transferability of non-MDR isolates (6 out of 11 isolates). However, conjugation on solid surface seems to be more efficient than conjugation on broth. *E. coli* transconjugants expressed a
resistance phenotype against amoxicillin but not tetracycline. Co-transfer of all the resistance traits was not observed in either non-MDR or MDR isolates. A summary of conjugation results are in Table 6-2.

6.3.2 Detection of presence of β-lactamase genes

TEM was detected in 7 out of 11 of non-MDR isolates. Among these isolates, TEM was present in only 1 (out of 5) of ESBLs isolates (Table 6-3). TEM was also present in all MDR isolates but not in the control isolate. No SHV or PSE-1 were detected in non-MDR, MDR or control isolates.

6.3.3 Detection of mutations of resistance genes

No mutation of all QRDR genes was detected in non-MDR and control isolates. However, in MDR isolates, regardless of the treatment drug, the presence of 2 mutations of gyrA at codons 83 and 87 and 2 mutations of parC at codons 80 and 84 was detected in all MDR isolates compared to control isolate and wild-type E. coli strain W3110. However, no mutations in gyrB were found in non-MDR, MDR or control isolates. Summary of the mutations in each isolate are in Table 6-4.

For mutations in gyrA, the mutation at codon 83 was a $C\rightarrow T$ transition in the codon TCG, resulting in the substitution of leucine for serine, while mutation at codon 87 was a $G\rightarrow A$ transition of codon GAC, leading to the substitution of asparagine for
aspartate. For \textit{parC}, a mutation at codon 80 was from a G→T transversion of codon AGC, resulting in the substitution of isoleucine for serine and a mutation at codon 84 was a substitution of alanine for glutamate from a A→C transversion of codon GAA.

AcrR were detected in all isolates. However, a mutation was detected at codon 7 in a non-MDR isolate (1 out of 11 non-MDR) with C→A transversion of codonCAA, but not in other isolates or control. A mutation was detected in \textit{soxS} of all MDR isolates at codon 12 with G→T transversion of codon GCA, leading to serine substitution for alanine. No \textit{soxS} mutations were detected in non-MDR or control isolates.

### 6.4 Discussion

In this study, both broth and solid conjugation methods were used to investigate and assure the maximal transferability of resistance genes in both non-MDR and MDR isolates. The results suggested that plate (solid) method is more sensitive than broth method. This could partly be due to the longer mating time in the plate method (overnight) compare to the broth method (1 h). However, only about half of non-MDR isolates (associated with amoxicillin treatment) transferred resistance by conjugation, whereas the other half did not. In general, amoxicillin resistance is mainly mediated by horizontal transfer of β-lactamase enzymes via the mobilisable DNA such as plasmids, integrons or conjugative transposons.\textsuperscript{7,15,22} These mobile DNAs, once enter the recipient cell, can integrate into chromosomal DNA of the recipient cell, suggesting a possibility of the negative results of conjugation in the other half of non-MDR isolates.\textsuperscript{23} In addition,
these non-MDR isolates may have other mechanisms of β-lactam resistance, besides the horizontal transfer of β-lactamases, such as decreased expression or the structural alteration of porin proteins or the efflux pumps. No conjugation detected in MDR isolates also indicates the different mechanisms of resistance, but not horizontal transfer.

Presence of TEM β-lactamase in the majority of both non-MDR and MDR isolates, was detected by PCR with primers that was designed from TEM-1 sequences. However, these results can only indicate the presence of TEM, but not specific to TEM-1. Since an amino substitution in TEM-1 could lead to varieties of TEM enzymes, in which most of them represent ESBLs, therefore, further tests such as sequencing need to be performed to characterize the presence of mutation compare to wild-type TEM-1 before indicating the specific TEM in these isolates.7-8,15,24

Nevertheless, Livermore (2008) has proposed a TEM characterization based on the hydrolytic profiles and MICs to cefotaxime and ceftazidime. These include TEM-3 (a 3 amino acid substitution of TEM-1), with high MICs to cefotaxime and ceftazidime (MICs of 64-256 and 32-128 respectively), TEM-10 (2 amino acid substitutions), exhibiting low-level resistance (MIC 0.25-4) to cefotaxime but high resistance (MIC 32-256) to ceftazidime, and TEM-12 (1 amino acid substitution) exhibiting susceptibility (MIC 0.12) to cefotaxime and increased resistance (MIC 4-8) to ceftazidime.24 Based on Livermore’s theory, TEM in non-MDR isolates susceptible to ceftiofur and all MDR isolates regardless of treatment, except in isolate 81, belongs to TEM-1 (group 2b β-lactamase enzymes).7,24 Isolate 81 is resistant to inhibition by clavulanic acid and extremely resistant to multiple drugs including gentamicin and
sulfamethoxazole/trimethoprim. TEM in isolate 81 is more likely to be TEM-12, which
belongs to group 2be β-lactamases, although its MIC for cefotaxime (MIC = 4) is a
higher than the range proposed by Livermore.\textsuperscript{7,24}

No PSE-1 were detected in any isolates, suggesting an absence of group 2c β-

lactamases.\textsuperscript{7} When taken together with ESBLs data (from chapter 5) and conjugation
results (Table 6-2 and Table 6-3), it is suggesting that β-lactamases of non-MDR isolates
which are susceptible to ceftiofur are more likely to be group 2b, TEM-1. Non-MDR
isolates that are resistant to ceftiofur express ESBLs which are mediated by group 2be β-
lactamases with other TEM enzymes; TEM-3 to TEM-26, but not TEM-1. β-lactamases
of MDR isolates are more likely to be TEM-1 that are integrated into the chromosomal
DNA.

For mutations in QRDRs genes, our data demonstrated that 2 mutations in $\text{gyrA}$
and 2 mutations in $\text{parC}$ are associated with the high level resistance to enrofloxacin
(MIC $\geq 32$) in all MDR isolates, supporting the previous studies in this area.\textsuperscript{19,25}
However, in all MDR isolates, the amino acid substitution of codon 84 in $\text{parC}$ is alanine,
which has never been reported. In $\text{E. coli}$, mutation at codon 84 of $\text{parC}$ is generally
caused by a substitution for glutamate by glycine, lysine, valine, tyrosine, or isoleucine.\textsuperscript{25,27}
However, amino acid substitution of alanine for glutamate at codon 84 of $\text{parC}$ has
been reported in $\text{E. faecalis}$.\textsuperscript{26}
Table 6-2 Conjugation results and frequencies of non-MDR and MDR isolates from 2 conjugation methods

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<sup>a</sup> A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin

<sup>b</sup>ND = not determined
Table 6-3 Presence of β-lactamases in non-MDR and MDR isolates

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<sup>a</sup> A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin
Table 6-4 Mutations and amino acid changes detected in non-MDR and MDR isolates

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<sup>a</sup> A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin
In \textit{soxS} gene, our MDR isolates demonstrated the amino acid substitution of serine for alanine at codon 12. SoxS is a general activator of SoxRS (superoxide response) regulon. SoxR protein, a transcriptional activator of SoxRS regulon, is activated by oxidation or nitrosylation to trigger transcription of \textit{soxS} gene. Through increased expression, SoxS protein is a direct activator for resistance to antibiotics.\textsuperscript{28} SoxS regulates 65-80 genes in \textit{E. coli}.\textsuperscript{29-31} Among these, genes for efflux pumps \textit{micF} and \textit{acrAB} are the major effectors of SoxRS-mediated antimicrobial resistance.\textsuperscript{32} Increased \textit{soxS} expression leads to down-regulation of outer membrane porin OmpF, resulting in decreased cell permeability. It also induces expression of acrAB-encoded efflux pump.\textsuperscript{28, 33-34} These combinations result in moderate MDR, which is a hallmark of SoxRS-mediated mechanism.\textsuperscript{32} Point mutations in \textit{soxR} gene could lead to constitutive expression of \textit{soxS}\textsuperscript{32}, however, no information is available regarding mutation in \textit{soxS}. SoxS mutation in our MDR isolates could possibly lead to constitutive expression of SoxRS, inducing expression of AcrAB-encoded efflux pump and downregulating OmpF protein.

Mutation of \textit{acrR} was detected in only 1 non-MDR isolate that exhibited ESBL and co-resistance to tetracycline. AcrR is a repressor of AcrAB-TolC system which is an important chromosomally encoded efflux pump in \textit{E. coli}.\textsuperscript{22, 26, 35-36} Mutation of \textit{acrR} has been reported contribute to high level FQ resistance in clinical \textit{E. coli} associated with mutations in QRDRs because of subsequent increased expression of AcrA.\textsuperscript{35} AcrA is a periplasmic membrane fusion protein that links inner and outer membrane proteins, mediates expression of inner membrane transporter AcrB and outer membrane channel TolC, and helps with the transport of drugs through AcrAB-TolC efflux pump.\textsuperscript{26, 37-38}
Mutation in 1 non-MDR isolate in our study may impact the ESBLs and tetracycline co-resistance, however, the mechanism needs to be further studied.

6.5 Conclusion

In this study, we demonstrated that amoxicillin therapy is associated with non-MDR. Resistance to β-lactam drugs is transferrable horizontally and mediated primarily by TEM β-lactamase enzyme. One exception occurred in the isolates that also exhibited an ESBLs. Enrofloxacin therapy, on the other hand, is associated with MDR, including resistance to β-lactams. Fluoroquinolone resistance associated with enrofloxacin treatment is mediated by double mutations in both gyrA and parC genes of gyrase and topoisomeraseIV enzymes, respectively. β-lactam resistance in MDR is mediated, partly, by non-transferrable TEM β-lactamase enzyme. In addition, mutation of a global regulator SoxS may impact MDR in fecal E. coli mediated by enrofloxacin therapy.

6.6 References


CHAPTER 7

SEROTYPES AND PATHOGENICITY ASSOCIATED WITH ANTIMICROBIAL-INDUCED RESISTANCE IN FECAL Escherichia coli OF HEALTHY DOGS

7.1 Introduction

*Escherichia coli* is the predominant facultative anaerobe in the normal intestine of both humans and many warm-blooded animals.\(^1\)\(^-\)\(^2\) Enteric *E.coli* are mostly nonpathogenic, however some strains can induce enteric disease through virulence factors.\(^3\)\(^-\)\(^4\) In general, pathogenic *E. coli* has been classified regarding to virulence factors and enteric disease into 6 groups: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic or Verotoxigenic *E. coli* (EHEC or VTEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), Uropathogenic *E. coli* (UPEC) and diffusely adherent *E. coli* (DAEC).\(^5\)\(^-\)\(^6\) In dogs, a variety of *E. coli* strains including attaching and effacing *E. coli* (AEEC), necrotoxigenic *E. coli* (NTEC), ETEC and VTEC have been associated with diarrheal diseases.\(^7\)

Fecal *E. coli* from healthy dogs have been reported to poses virulence attributes regarded as markers of enteropathogenicity, such as *eae* and *sta* genes, heat-stable enterotoxin, verotoxin, and a hemolysin.\(^6\)\(^,\)\(^8\)\(^-\)\(^10\) These findings suggest that potential pathogens are carried by healthy dogs.\(^9\)
Resistance and virulence are often perceived to emerge simultaneously. Recently, some studies have suggested that resistance to antimicrobial drugs, such as quinolones, ampicillin, tetracycline and multidrug resistance (MDR), is associated with a reduced potential for invasiveness and a decrease in the presence or the expression of some virulence factors in *E. coli*.\textsuperscript{11-20} However, the mechanism underlying these findings are not well understood.

The purpose of this study was to examine the relationship between resistance and the presence of virulence factors in fecal *E. coli* isolates with multidrug resistance (MDR) versus those not expressing multidrug resistance (non-MDR) in response to antimicrobial therapy in healthy dogs. We have previously demonstrated that close to 100% of fecal *Escherichia coli* in healthy dogs develop non-MDR versus MDR when treated with amoxicillin (10 mg/kg bid) or enrofloxacin (5 mg/kg sid), respectively, with no resistance emerging in untreated controls (n=8 dogs per treatment group). With this study, isolates with similar phenotypes and genotypes were subjected to serotyping and serotypes were subsequently matched with resistance patterns.

### 7.2 Materials and Methods

#### 7.2.1 Fecal *E. coli* isolates

A total of 18 MDR and non-MDR fecal *E. coli* isolates, each represents each genotype within each phenotype, were used in this study. All isolates received from our previous study by the following methods.
Twenty-four healthy, antimicrobial-free, purpose-bred adult hound dogs were studied. All dogs were maintained with regular adult maintenance diet (Hill’s® Science Diet® Adult Large Breed dry food, Hill’s Pet Nutrition Inc., Topeka, KS). Each dog was randomly housed in individual, climate controlled cage in a strictly-controlled environment, as well as kennel access limitation to minimize mechanical transmission of microbes and antimicrobial resistance genes. Dogs were randomly divided in three groups of eight. Each group received a different antimicrobial therapy: group1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 h, group2 (G2) was treated with 5 mg/kg enrofloxacin orally every 24 h, and Group3 (G3) received no treatment and was reserved as control group. Both drugs were administered for 7 to 21 days, with the duration based on the emergence of antimicrobial resistance, defined as a proportion of total resistant *E. coli* to total *E. coli* counts ≥75%. The time at ≥75% resistance was defined as time of resistance (T), and then antimicrobial therapy was discontinued.

Fresh fecal samples were obtained by digitally collected *per rectum* into sterile containers prior to (Baseline; B) and every 3 days during treatment until time of resistance (T). Drug was then discontinued and monitoring continued weekly up to 4 weeks, or until the resistance resolved (≤25% of cfu resistant or study end; E), whichever came first. If the resistance did not resolve by 4 weeks post-therapy, E would be reported as 4 weeks.

Antimicrobial resistant *E. coli* were obtained randomly from the 18-24 h growth of fecal dilutions (with 0.9% saline solution) at 37°C on CHROMagar® *E. coli* agar plates (CHROMagar, Paris, France) containing either amoxicillin or enrofloxacin at
concentrations 1 tube dilution below the breakpoint MIC (MICBP) of either drug: at 16 µg/ml agar for amoxicillin and 2 µg/ml agar for enrofloxacin respectively. Ten representative antimicrobial susceptible isolates were obtained from fecal samples of control, untreated dogs, at baseline (B; D0).

7.2.2 MDR and non-MDR fecal *E. coli* isolates

From each of the three time points (B, T or E) for each group of dogs, 10 *E. coli* colonies were randomly selected from the population of antimicrobial resistant *E. coli* recovered from dogs receiving either amoxicillin (n=8 dogs) or enrofloxacin (n=4 dogs), with a total of 180 isolates. Levels of resistance (susceptibility) to amoxicillin and enrofloxacin, and type of resistance were performed using Etest® (Epsilometer or Epsilon; AB Biodisk/ BioMérieux, Inc. Hazelwood, MO) strips and Vitek® automated system (BioMérieux, Inc. Hazelwood, MO) with Gram Negative Veterinary Susceptibility Test Cards (GNS-207) based on the susceptibility to 8 antibacterial classes; 17 antimicrobial drugs including Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Carbenicillin, Ceftazidime, Ceftiofur, Cephalothin, Chloramphenicol, Ciprofloxacin, Enrofloxacin, Gentamicin, Nitrofurantoin, Piperacillin, Tetracycline, Ticarcillin, Tobramycin, and Trimethoprim/Sulfamethoxazole, respectively. Multidrug resistance (MDR) was indicated if *E. coli* was resistant to 3 or more unrelated drug classes.

Genomic fingerprints were determined in all 190 antimicrobial resistant isolates using Pulse-field gel electrophoresis (PFGE) in accordance with the Pulse-Net
standardized protocol for molecular subtyping of E. coli O157:H7. Analysis of band patterns and construction of dendrograms were performed using the Dice correlation coefficient and clustering of patterns was performed by unweighted pair group with arithmetic averaging (UPGMA) with 90 percent of similarity between patterns to address the genetic relatedness among the PFGE patterns.

7.2.3 Serotype and virulence gene determination

Eighteen representative fecal E. coli isolates were randomly selected from each phenotype and genotype. Serotype and virulence gene determinations were performed at the Pennsylvania State University E. coli Reference Center, Pennsylvania. Briefly, serum agglutination assay was used to screen isolates for the presence of 187 O antigens and 52 H antigens. The isolates were also examined for the presence of genes that code for Heat labile toxin (LT), Heat stable toxins (STa, STb), Shiga-like toxins (stxI, stxII), Cytotoxic necrotizing Factors (cnf1, cnf2), and intimin (eaeA).21

7.3 Results

7.3.1 Serotypes

Seven serotypes were detected in 11 non-MDR isolates (63.63%) from amoxicilllin treatment, whereas one serotype was detected in 3 MDR isolates (100%) from enrofloxacin treatment (Table 7-1). The serotype of MDR E. Coli (O9:H30) was not
similar to those of non-MDR isolates (O83:H31, O8:H30, O138:H43, O-negative:H12, O-negative:H32, O11: H11or 47, and O-negative:H-positive). Control (susceptible) isolates exhibited serotype O5:H4 which also was different from both non-MDR and MDR serotypes.

7.3.2 Virulence factors and toxins

Among the virulence genes tested, only cytotoxic necrotizing factors (CNF) 1 and 2 were detected. Presence of \textit{cnf} 1 gene was in association with 2 phenotypes: in 1 control isolate (O4:H5), and in 2 non-MDR isolates from amoxicillin therapy that had the similar serotype (O83:H31). Presence of \textit{cnf} 2 was in association with O-negative:H-positive isolates. No other toxins or adhesins were expressed in isolates of this study.

7.4 Discussion

Serotypes of \textit{E. coli} from feces of healthy, non-diarrheic dogs have been less studied compared to those of urinary tract infections. Most of the fecal \textit{E. coli} serotype data were reported as controls in comparisons with serotypes of urine or blood from urinary tract infection hosts. In this study, we have demonstrated the various serotypes from fecal \textit{E. coli} of healthy, non-diarrheic dogs. With one exception (O138 in our study), each of the 7 distinct O-serotypes demonstrated in this study; O4, O8, O9, O11, O83, O-negative, have been reported as the dominating serotypes in fecal and extra-
intestinal *E. coli* of healthy dogs and cats, in which O4 and O6 were the most frequently found.\(^7,22\)

Among these 7 distinct O-serotypes, 9 serotypes were detected based on O and H antigens, with most of them (7 serotypes) found in non-MDR isolates compared to MDR and control isolates. In non-MDR isolates, serotypes were varied (6 different serotypes) among the phenotypes that were resistant to tetracycline, whereas only one serotype, O83:H31, was detected in non-MDR isolates susceptible to tetracycline (resistance to β-lactams including ceftiofur and nitrofurantoin). Interestingly, only one serotype, O9:H30, was detected in all MDR isolates regardless of antimicrobial treated. This clone might be related to enrofloxacin resistance, possibly due to the selection pressure of enrofloxacin resistance colonies. In control (susceptible) isolate obtained from non-treated dog, 1 serotype, O4:H5, was detected. This serotype is also similar to that reported in feces and extra-intestinal samples of both dogs and cats.\(^7\)

From all 18 isolates determined for virulence factors, only cytotoxic necrotizing factors 1 and 2 (*cnf*1 and *cnf*2) were detected. *cnf*1 was present only in control and non-MDR isolates susceptible to tetracycline (serotypes O4:H5 and O83:H31, respectively), while *cnf*2 was present in isolates from a non-MDR phenotype, O-negative:H-positive serotype. CNFs, especially *cnf*1, have been reported in fecal *E. coli* obtained from both healthy and diseased dogs.\(^6,22-25\)
Table 7-1 Serotypes and virulence factors in non-MDR, MDR and susceptible *E. coli*

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<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype</th>
<th>MDR&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> A = ampicillin; B = carbenicillin; P = piperacillin; R = ticarcillin; L = cephalothin; X = amoxicillin/clavulanic acid; C = ceftiofur; F = ciprofloxacin; E = enrofloxacin; T = tetracycline; G = gentamicin; S = trimethoprim/sulfamethoxazole; H = chloramphenicol; N = nitrofurantoin

<sup>b</sup> MDR is defined as resistance to 3 or more unrelated drug classes

LT = Heat labile toxin (ETEC), STa, STb = Heat stable toxin (ETEC), stx1, stx2 = Shiga-like toxin (EHEC), cnf1, cnf2 = Cytotoxic necrotizing factor (NTEC), eae = Intimin gamma (EHEC)
Extra-intestinal pathogenic *E. coli* strains from dogs and cats, such as uropathogenic *E. coli* (UPEC), are frequently found to be associated with a few O-serotypes, such as O2, O4, O6, O8, O9, O22, O25, O75 and O83, which are also frequently found in normal fecal flora of dogs and cats. However, UPEC differ from commensal intestinal *E. coli* due to the presence of specialized virulence factors (uropathogenic virulence factors; UVFs) such as P-fimbriae and produce alpha-hemolysin and a necrotizing cytotoxin (CNF1) that promote extraintestinal infection. The CNF-producing strains with negative for other virulence markers associated with diarrheal disease are not indicative of entero-pathogenicity, but could be closely associated with extraintestinal infections. In our study, however, none of the dogs were sick nor had either diarrhea or urinary tract infection. More importantly, no P-fimbriae, hemolysin, other UVFs were detected in our *E. coli* strains, indicating that our *E. coli* stains are commensal *E. coli*, not UPEC. In addition, our *cnf*1 finding in a control isolate with serotype O4:H5 also supports the studies of Blanco et al (1993) and Beutin et al (1999) that O4 and O6 serotypes were the most frequent found among *cnf*1 strains in dogs and cats.

Although, our data came from fecal *E. coli* of healthy dogs, which are mostly non-pathogenic that tend to exhibit less or no virulence factors, virulence factor *cnf*1 was detected in control (susceptible) isolate, but not in most of the resistance isolates, particularly MDR. Only one serotype emerged in isolates expressing resistance to enrofloxacin. This may indirectly support the recent theory that resistance to antimicrobial drugs, especially quinolones, is associated with a significant decrease in inferred virulence in *E. coli*. 
7.5 Conclusion

Our data demonstrate the various serotypes in normal fecal *E. coli* in dogs, with more variety in non-MDR than that in MDR and control. Serotype clonality seems to be related to enrofloxacin resistance isolates regardless of the treatment drug or co-resistance, but not related to amoxicillin resistance. In addition, antimicrobial resistance of fecal *E. coli*, either non-MDR or MDR, induced by either amoxicillin or enrofloxacin treatment in dogs was not associated with an increase in pathogenicity, i.e. virulence factors. This may indirectly support the possibility of negative associations between antimicrobial resistance and presence of virulence factors in *E. coli*.

7.6 References


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CHAPTER 8

GENERAL CONCLUSIONS

Because of the rapidly increasing antimicrobial resistance problem, it has become important to decrease the inappropriate use of antimicrobials and also to optimize the dosing regimens to prevent resistance development. In these studies we have demonstrated that certain antibiotic concentrations can select for resistant, not only in the pathogenic but also in the commensal bacteria.

Our data demonstrated the factors that have significant impact on the transmission of antimicrobial resistance genes among fecal coliforms which include source of animals, environmental conditions as well as animal husbandry. However, their specific roles in this matter have yet to be determined.

We have demonstrated that the administration of either amoxicillin or enrofloxacin, each commonly used to treat *E. coli* infections in dogs, is associated with rapid emergence of high level antimicrobial resistance to the drug in fecal *E. coli*. Characteristics of resistance, including phenotypic patterns and mechanisms of resistance varied with the treatment drug.

For amoxicillin treated dogs, resistance emerged in 90-96% of isolates in an average of 7 days, and resolved within 7 days of discontinuing therapy. Most notably, enrofloxacin, but generally not amoxicillin, was associated with MDR. These changes
occur when either drug is used according to recommended dosing regimens. Resistant isolates emerging with amoxicillin treatment were characterized by variable phenotypes and PFGE genotypes. Resistance associated with amoxicillin was largely, but not exclusively limited, to β-lactam drugs. These included penicillins, selected cephalosporins but not carbapenems. Further, amoxicillin treatment was associated with emergence of ESBLs in some, but not all *E. coli* isolates. Horizontal resistance was associated with β-lactamase enzymes such as TEM. Resistance did not appear to involve SHV or PSE-1. Although selected isolates expressed ESBL, this resistance was not transferable.

In enrofloxacin treated dogs, resistance involved 100% of fecal *E. coli*, emerged in 9 days, and in those dogs for which *E. coli* was still detected, resolved in 10.5-21 days. In contrast to amoxicillin, enrofloxacin therapy was associated with complete eradication of *E. coli* in 4 dogs. As with amoxicillin therapy, resistance to enrofloxacin in enrofloxacin treated dogs was high level. In contrast to amoxicillin therapy, however, isolates expressed MDR, with fewer genotypes but more variable phenotypes among genotypes compared to the amoxicillin-treated dogs. Resistance mechanisms that were studied were non-transferrable. Resistance to fluoroquinolones induced by enrofloxacin was mediated by mutations in both *gyrA* and *parC* genes of gyrase and topoisomerase IV enzymes, with double mutations in each gene. Resistance to amoxicillin and other β-lactam drugs was mediated, in part, by TEM β-lactamase enzyme, but not SHV or PSE-1. Mechanisms of beta-lactam resistance did not involve ESBLs. Mutation of a global regulator SoxS was detected that might impact MDR in fecal *E. coli* mediated by...
enrofloxacin therapy. At no time was resistance detected in control animals that received no drug.

Our findings also demonstrate the various serotypes in normal fecal *E. coli* in dogs, with more variety in non-MDR than that in MDR and control. Serotype clonality seems to be related to enrofloxacin resistance isolates regardless of the treatment drug or co-resistance, but not related to amoxicillin resistance. In addition, antimicrobial resistance fecal *E. coli*, either non-MDR or MDR, induced by either amoxicillin or enrofloxacin treatment in dogs was not associated with an increase in pathogenicity, i.e. virulence factors. This may indirectly support the possibility of negative associations between antimicrobial resistance and presence of virulence factors in *E. coli*.

Finally, these studies suggest that use of all antimicrobials should be judicious, not only to pathogenic but also to commensal bacteria, but caution is indicated for fluoroquinolones in particular such that emergence of MDR *E. coli* might be avoided. Genotypic characterization and mechanisms of resistance in non-MDR versus MDR isolates need to be further studied to explain, more in depth, the impact of antimicrobial therapy on the advent of antimicrobial resistance and the mechanism of resistance genes transfer in commensal *E. coli* in dogs.