

# Impact of Routine Antimicrobial Therapy on Canine fecal *Escherichia coli* Antimicrobial Resistance: A Pilot Study

Dawn M. Boothe, DVM, PhD  
DACVIM (Internal Medicine), DACVCP  
Nipattra Debavalya, DVM, MS, PhD<sup>2</sup>

<sup>1</sup>American College of Veterinary Clinical Pharmacology,  
Clinical Pharmacology  
College of Veterinary Medicine  
Auburn University

<sup>2</sup>Department of Veterinary Pharmacology  
Faculty of Veterinary Science  
Chulalongkorn University.

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## CLINICAL RELEVANCE

The impact of 7 days of amoxicillin (G1; PO 10 mg/kg b.i.d.; n=2), or enrofloxacin (G2; 5 mg/kg PO once daily; n=2) on fecal *E. coli* was studied in six healthy dogs (G3= no treatment, n=2). Total coliform count, percent resistant *E. coli*, presence of multidrug resistance (MDR), and MIC<sub>90</sub> were studied before, during and 21 days after therapy. By day 3, approximately 100% of *E. coli* expressed high-level resistance to the treatment drug. Resistance was high-level and for enrofloxacin, was associated with MDR and persisted to study end. This study demonstrates a mechanism whereby rapid, high-level and for enrofloxacin, MDR associated antimicrobial resistance emerges during routine therapy.

## ABSTRACT

Increased prevalence of antimicrobial resistance in various bacterial species from pet animals has been reported in the United States and the United Kingdom, with resistance generally associated with antimicrobial therapy. Increased prevalence of antimicrobial resistance in various bacte-

rial species from pet animals has been reported in the United States and the United Kingdom, with resistance generally associated with antimicrobial therapy. This study was performed in order to investigate the feasibility of a larger scale study that would focus on the impact of antimicrobial therapy on the fecal flora of normal dogs. Either amoxicillin or enrofloxacin administered at recommended dosing regimens is associated with rapid development of high level antimicrobial resistance to that drug by the majority of fecal coliform, and particularly *E. coli*. Resistance associated with amoxicillin resolved when therapy is discontinued. In contrast, resistance to enrofloxacin persisted.

## INTRODUCTION

Emergence of antimicrobial resistance is an increasing concern in both human and veterinary medicine.<sup>1,2</sup> Increased prevalence of antimicrobial resistance in various bacterial species from pet animals has been reported in the United States and the United Kingdom, with resistance generally associated with antimicrobial therapy.<sup>1-5</sup> The role of previous antimicrobial therapy in the emergence of resistance is generally accepted, as is the role of selection pressure.<sup>6</sup> Use of antimicrobials selects for resistance in com-

mensal as well as pathogenic bacteria.<sup>5-8</sup>

However, direct evidence of this relationship is limited, being largely based on epidemiologic examination of cause and effect relationships in resistance patterns and antimicrobial use. Although a number of animal models have been developed to investigate antimicrobial-induced resistance, few studies have focused on companion animals. One of the long-term objectives of our laboratory is understanding the role of routine antimicrobial therapy in the emergence of resistant microorganisms in the canine or feline patient.

*Escherichia coli* is a common inhabitant of the intestinal tracts of animals and humans among the gastrointestinal commensal flora.<sup>9-10</sup> Fecal *E. coli* is frequently used to represent the intestinal flora due to the ease and convenience of sample collection in live animals.<sup>7, 11</sup> In addition, fecal *E. coli* is considered as a very good indicator for selection pressure by antimicrobial use.<sup>7</sup> Amoxicillin and enrofloxacin are among the most commonly used antimicrobials in veterinary medicine. Amoxicillin is a semi-synthetic  $\beta$ -lactam, while enrofloxacin is a synthetic fluoroquinolone (FQ).<sup>12-16</sup> 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.

## MATERIALS AND METHODS

### Animals and Sample Collection

Six healthy, antimicrobial-free, purpose-bred adult hound dogs were randomly divided in three groups of two: group1 (G1) was treated with 10 mg/kg amoxicillin orally every 12 hR, group2 (G2) with 5 mg/kg enrofloxacin orally every 24 hR, 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, which began at least 180 days prior to the study, and each dog was randomly housed in individual, climate-controlled cage in close proximity to each other. Access of personnel to the ken-

nel was restricted to minimize mechanical transmission of microbes and antimicrobial resistance genes.

### Total and resistant coliform counts

Fresh fecal samples were collected per rectum from each dog into sterile containers prior to (D0), days (D) 1, 3, 5, 7 of treatment, and days (P) 3, 7, 14 and 21 post-therapy. Gloves were changed between dogs. All samples were collected on the same time of each sampling day, within 1 hr post-feeding. Fecal samples were processed within 2 hr of collection.

Serial 10-fold dilutions were prepared from 1 g fecal sample in 0.9% sodium chloride solution. For each dilution, 0.1 ml was transferred onto MacConkey agar plates for total coliforms counts (TCC). For total resistant coliform counts (TRCC), plates were supplemented with either amoxicillin or enrofloxacin at concentrations one tube dilution below the respective resistant breakpoint MIC ( $MIC_{BP}$ ) for each drug as set by the Clinical Laboratory Standards Institute (CLSI) for gram negative bacteria in Veterinary Medicine: 2  $\mu$ g/ml for enrofloxacin ( $MIC_{BP}$  4  $\mu$ g/ml) and 16  $\mu$ g/ml for amoxicillin ( $MIC_{BP}$  32  $\mu$ g/ml), respectively.<sup>17</sup> 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<sub>10</sub> cfu per gram wet fecal weight. Antimicrobial resistant coliform counts were expressed as the proportion of TRCC of each drug to TCC ( $[TRCC/TCC] \times 100$ ).

### *E. coli* Identification and Level of Antimicrobial Resistance

Based on morphology,<sup>18 10</sup> presumed *E. coli* colonies resistant to treatment drug at each time-point were randomly selected from antimicrobial containing MacConkey agar plates. Isolates were confirmed to be *E. coli* by screening with Kovacs tests (Remel/Thermo Fisher Scientific, Lenexa, KS).

**Table 1.** Antimicrobial drugs, drug classes and concentrations ( $\mu\text{g/ml}$ ) determined on Vitek<sup>®</sup> Gram Negative Veterinary Susceptibility Test Cards (GNS-207) and MICBP ( $\mu\text{g/ml}$ ) for resistance of each drug based on CLSI guideline (M31-S1)<sup>14</sup>

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

Isolates were then tested for susceptibility to amoxicillin and enrofloxacin using E-test<sup>®</sup> (Epsilon meter or Epsilon; AB Biodisk/BioMérieux Inc., Hazelwood, MO) strips according to the manufacturer's instructions and CLSI interpretive guidelines.<sup>19</sup> Briefly, each 18 to 24 hr 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 E-test<sup>®</sup> strips were 0.016 to 256  $\mu\text{g/ml}$  for amoxicillin, and 0.002 to 32  $\mu\text{g/ml}$  for enrofloxacin. All plates

were incubated at 37°C for 18 to 24 hr. The results (MICs) were determined on the basis of the intersection of the elliptical zone of growth inhibition with the MIC scale on each E-test<sup>®</sup> strip and expressed as the MIC required to inhibit the growth of 90% of the isolates (MIC<sub>90</sub>).

#### **Type of Antimicrobial Resistance**

Five out of each 10 antimicrobial resistant isolates subjected to E-test<sup>®</sup> to determine the level of resistance were randomly selected and subjected to susceptibility testing to 17 different drugs using Vitek<sup>®</sup> automated system (BioMérieux Inc, Hazelwood, MO) with Gram Negative Veterinary Susceptibility Test Cards (GNS-207). The drugs for which MICs were determined represented eight drug classes (Table 1) and included gentamicin, amikacin and tobramycin,

**Table 2** Mean  $\pm$ SE of log transformed total coliform counts (TCC) in each group (n=2 per group)

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

(aminoglycosides), amoxicillin/clavulanic acid, ampicillin, carbenicillin, piperacillin, ticarcillin, ceftazidime, ceftiofur, and cephalothin (beta-lactams), chloramphenicol (phenicols), Ciprofloxacin and enrofloxacin (fluoroquinolones), nitrofurantoin, tetracycline, and trimethoprim/aulfamethoxazole.

Testing was implemented according to the manufacturer's instructions.<sup>20</sup> 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<sup>®</sup> DensiCheck (BioMerieux Inc, Hazelwood, MO). Then, 50  $\mu$ l 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 a vacuum before being loaded into the Vitek<sup>®</sup> machine. Results were analyzed and phenotypes of each resistant *E. coli* colony were expressed as susceptible (S) or resistant (R) based to each drug on CLSI MICBP of *E. coli* to each drug (M31-S1).<sup>17</sup> An isolate was considered to be expressing multidrug resistance (MDR) if resistance was expressed to 3 or more unrelated drug classes.<sup>21</sup>

### Pulse-field Gel Electrophoresis (PFGE)

In order to examine the clonal relationship of resistant isolates in each dog, three representative isolates were randomly selected from antimicrobial resistant *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 *E. coli* O157:H7.<sup>2,22</sup> 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  $\mu$ l of cell suspension, 20  $\mu$ l 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.

**Table 3.** Phenotypes, percent of isolates with that phenotype resistant to the treatment drug and the MIC 90 of those isolates as determined across time in dogs treated with either amoxicillin at 10 mg/kg orally twice daily, (G1), enrofloxacin 5 mg/kg orally once daily (G2), or no treatment (G3)

Dog	Time Point	Phenotypes <sup>a</sup>	Percent Resistant	MDR <sup>b</sup>	MIC90 (Etest)	
					Amoxicillin	Enrofloxacin
G1: Amoxicillin Treatment						
1,2	D0	ABPRXLS <sup>c</sup>	25%	N	≥ 512	0.094
1	D3	ABPRXLS	100%	N	≥ 512	0.094
2		ABPRLFETSH	67%	Y	≥ 512	≥ 64
2		ABPRXLFETSH	33%	Y	≥ 512	≥ 64
1	D5	ABPRXLS	100%	N	≥ 512	0.094
2		ABPRLFETSH	67%	Y	≥ 512	≥ 64
2		ABPRXLFETSH	33%	Y	≥ 512	≥ 64
1,2	D7	ABPRXLS	100%	N	≥ 512	0.094
1,2	P3	ABPRXLS	100%	N	≥ 512	0.125
1,2	P7	ABPRXLS	100%	N	≥ 512	0.125
1,2	P14	ABPRLSd	100%	N	≥ 512	0.125
1,2	P21	ABPRLS	25%	N	≥ 512	0.125
G2: Enrofloxacin Treatment						
3,4	D3	ABPRLFETSH <sup>f</sup>	10%	Y	≥ 512	≥ 64
		ABPRXLFETSH <sup>e</sup>	90%	Y	≥ 512	≥ 64
3,4	D5	ABPRLFETSH	60%	Y	≥ 512	≥ 64
		ABPRXLFETSH	40%	Y	≥ 512	≥ 64
3,4	D7	ABPRLFETSH	20%	Y	≥ 512	≥ 64
		ABPRXLFETSH	80%	Y	≥ 512	≥ 64
3,4	P3	ABPRLFETSH	10%	Y	≥ 512	≥ 64
		ABPRXLFETSH	90%	Y	≥ 512	≥ 64
3,4	P7	ABPRLFETSH	50%	Y	≥ 512	≥ 64
		ABPRXLFETSH	50%	Y	≥ 512	≥ 64
3,4	P14	ABPRLFETSH	40%	Y	≥ 512	≥ 64
		ABPRXLFETSH	60%	Y	≥ 512	≥ 64
3,4	P21	ABPRLFETSH	80%	Y	≥ 512	≥ 64
		ABPRXLFETSH	20%	Y	≥ 512	≥ 64
G3: No Treatment						
5,6	D5	ABPRXLS	55%	N	≥ 512	0.094
5,6	D7	ABPRXLS	55%	N	≥ 512	0.064

\*The absence of data for a time point indicates that no resistance was detected in fecal *E. coli* at that data point.

<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

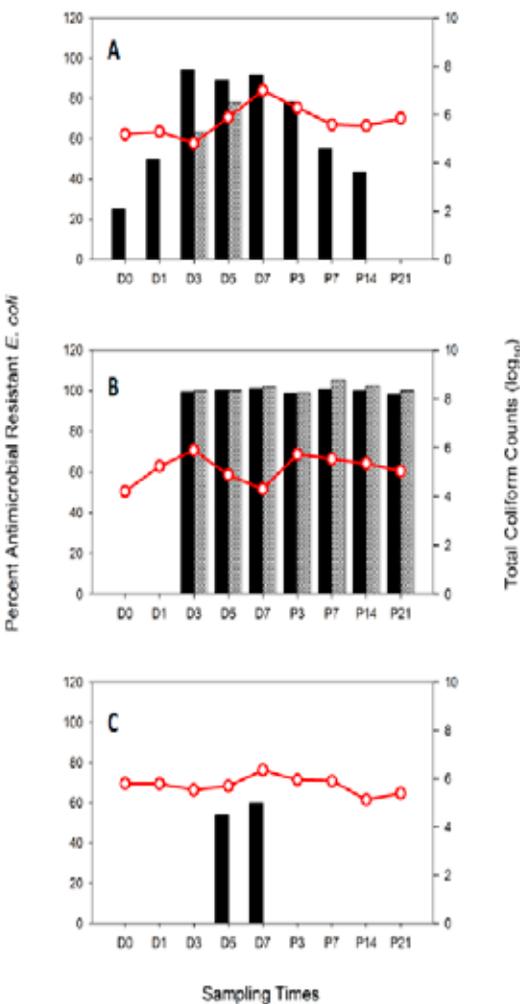
<sup>b</sup> MDR is defined as resistance to 3 or more unrelated drug classes

<sup>c,d,e,f</sup> Phenotypes 1,2, 3 and 4, respectively

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 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 hr, 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.

**Figure 1:** Total coliform counts (-o-) (with at least 90% being *E. coli*), percent amoxicillin resistant coliforms (■), and percent enrofloxacin resistant coliforms (▨) in dogs treated orally from days 1 through 7 with amoxicillin at 10 mg/kg twice daily (a; G1; n=2), enrofloxacin 5 mg/kg once daily (b; G2; n=2), or receiving no treatment (c, control, G3; n=2).



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### 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. Analysis of variance was used to compare colony counts across time.

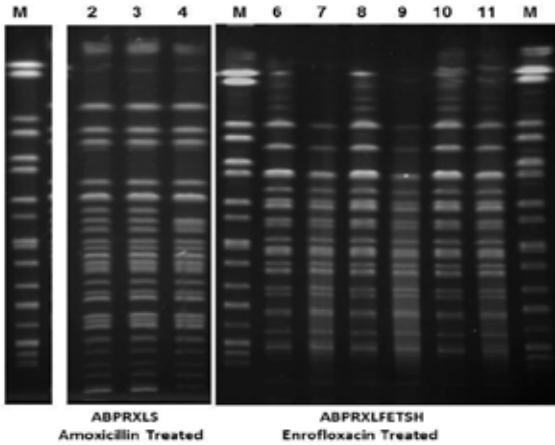
For PFGE analysis, Tiff images of the gels were normalized using the BioNumerics® software, version 4.5 (Applied Maths, Austin, TX). Analysis of band patterns and construction of dendrograms were performed using the Dice correlation coefficient and clustering of patterns was performed by unweighted pair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

### RESULTS

#### Total Coliforms Counts

Mean TCC are similar across time within and among the treatment

**Figure 2.** Example images of pulsefield gel electrophoresis of isolates expressing beta-lactam resistance in animals treated with amoxicillin (lanes 2-4, day 7 of treatment) and isolates expressing enrofloxacin resistance in animals receiving enrofloxacin (M=marker). The phenotype of each pulsotype is listed.



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

groups with all colony counts being with 2 log units (Table 2). TCCs were 104 - 106, 105 - 107, and 105 - 106 cells per gram feces in G1, G2, and G3 respectively.

**Percent Antimicrobial Resistance**

In the control group (G3), transient resistance to amoxicillin emerged on day 5 and 7; no resistance emerged to enrofloxacin. For the G1 (amoxicillin treatment), 25% of coliforms were resistant to amoxicillin at D0 (prior to treatment), but resistance was absent at baseline in G3 (Figure 1). In G1, resistance to amoxicillin gradually increased to close to 100% by D3 and returned to baseline by P21 (Figure 1A). Resistance to enrofloxacin was transiently present at D3 and D5 in both dogs. For G1 isolates, MIC<sub>90</sub> for amoxicillin during and post-treatment was >512 µg/ml, whereas the MIC<sub>90</sub> for enrofloxacin was 0.125. For G2 isolates, MIC<sub>90</sub> for enrofloxacin during and post-treatment was > 64 µg/ml and toward amoxicillin > 512 µg/ml. Enrofloxacin-

induced resistance yielded two phenotypes in G2. These isolates exhibited MDR with resistance being expressed to all drug classes except nitrofurantoin and aminoglycosides.

**Resistance Phenotypes**

A total of four different resistance phenotypes were recorded (Table 3) among all three treatment groups. Phenotype 1 (ABPRXLS) included resistance to beta-lactams, including amoxicillin-clavulanic acid, and trimethoprim-sulfamethoxazole resistance. This phenotype was consistently present in one, or more commonly, both G1 dogs throughout all time periods, including baseline. This phenotype (which also was transiently expressed in G3 dogs) increased from a baseline of 25% at baseline to 100% by day 7 of treatment with amoxicillin, but was absent by study end. However, by study end, the second phenotype (ABPRLS) emerged in both G1 dogs, differing from phenotype

1 only by the absence of resistance to amoxicillin-clavulanic acid (X). In G2, the third (ABPRXLFETSH) and fourth (ABPRLFETSH) phenotypes occurred. Both phenotypes included resistance to enrofloxacin, as well as resistance to all drug classes studied save the aminoglycosides (MDR; Table 3).

The difference between phenotypes 3 and 4 in G2 (as with the difference between phenotypes 1 and 2 in G1) was the absence of resistance to X. Both phenotypes 3 and 4 were absent at baseline in G2, but rapidly emerged in both dogs by day 3 of enrofloxacin treatment, persisting throughout the study period. Both of these phenotypes were transiently expressed in one G1 dog such that it temporarily replaced phenotype 1 in this dog. The control group (G3) remained free of resistant *E. coli* with the exception of transient resistance associated with phenotype 1 (beta-lactam and sulfonamide) in both

dogs on days 5 and 7 (Table 3).

### PFGE Pulsotypes

A total of 21 representative isolates (at least five isolates for each of the four phenotypes) were subjected to PFGE, genomic fingerprinting, and dendrogram analysis (Figure 2). From these 4 phenotypes, 8 pulsotypes emerged (Figure 2). Analysis revealed that phenotype (ABPRXLS) 1 consisted of one pulsotype (present in both G1 dogs, but also transiently in both G3 dogs). This pulsotype was related (90% homology) to all three related pulsotypes that comprised phenotype 2 (ABPRL; Figure 2). Thus, all isolates persisting in G1 dogs were related. Indeed, one pulsotype expressed both phenotypes 1 and 2 (not shown). Phenotype 3 (ABPRXLFETSH) consisted of two pulsotypes which were related to each other, but not to any other pulsotype. Phenotype 4 (ABPRLFETSH) consisted of three pulsotypes not related to any other pulsotype, including one another, despite being present in both G2 dogs (Fig. 2).

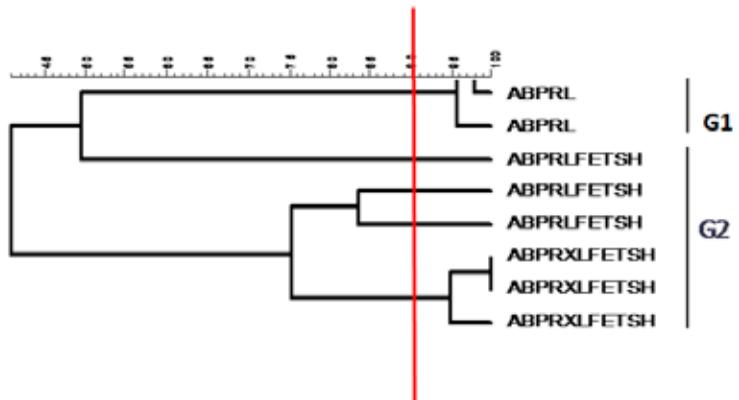
### DISCUSSION

This study was performed in order to investigate the feasibility of a larger scale study that would focus on the impact of antimicrobial therapy on the fecal flora of normal dogs. We studied purpose-bred dogs, housed in a restricted kennel such to enable the transmission of microbes and so that antimicrobial resistance genes might be minimized. Transient amoxicillin resistance emerged in D5 and D7 in control dogs (G3) but did not return

after this initial appearance. Further, enrofloxacin resistance emerged at D3 and D5 in amoxicillin-treated dogs (G1). However, in both groups of dogs, resistance resolved with the next sampling period and did not re-emerge.

This transient resistance may reflect mechanical transfer of resistance genes between dogs despite the implementation of preventative protocols. Our data demonstrated that neither amoxicillin nor enrofloxacin therapy had direct effects on the total coliform or *E. coli* counts. We chose to study *E. coli* as it is the major coliform (and Gram negative facultative anaerobe) in dog feces. Our findings disagree with other studies, in which FQ had profound a impact on the facultative anaerobic population of the gastrointestinal flora by transiently suppressing or eliminating microbes. Microbial resistance status returned to pre-antimicrobial administration numbers within 2 weeks after cessation of therapy. 24-25 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 such that they were undetectable by 3 days of therapy

**Figure 3.** Dendrogram of the representative antimicrobial resistance *E. coli* isolates from each of the 4 phenotypes from each group. Phenotypes are: 1 (ABPRXLS) and 2 (ABPRL) expressed predominantly by G1, and 3 (ABPRXLFETSH) and 4 (ABPRLFETSH) expressed predominantly by G2.



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

and remained suppressed throughout 2-week study period. Upon termination of enrofloxacin treatment, fecal coliforms gradually returned to levels comparable to those seen prior to antibiotic treatment by 8 days.<sup>26</sup>

This is in contrast to our study for which total coliform and *E. coli* counts did not appear to be impacted by drug therapy. The differences between the two studies might reflect, in part, high variability 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 approximated baseline by 3-week post-treatment. We feel that the transient enrofloxacin resistance that occurred in G1 dogs on day 3 and day 5 may reflect mechanical transmission dogs treated with enrofloxacin developed resistance in all measurable fecal *E. coli* by day 3 of enrofloxacin therapy. Further, all isolates were characterized by MDR. However, neither enrofloxacin resistance nor its associated MDR resolved during the 3-week post-treatment study period. This suggests that different mechanism of resistance emerged for G1 and G2 dogs such as mutations, which are reproduced in daughter cells, compared to plasmids which are exchanged. Further, enrofloxacin induced resistance to multiple classes of antimicrobials for which resistance mechanisms differ from that for fluorinated quinolones.

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.<sup>27-29</sup> However, a decrease of FQ concentration in the bacterial cell also contributes to resistance through expression of efflux pumps such as AcrAB-TolC efflux system.<sup>30</sup> However this efflux system

mediates resistance to several drugs including FQ, ampicillin, tetracycline, chloramphenicol, rifampicin, and puromycin.<sup>29,31</sup> Horizontal transfer of FQ resistance genes by either plasmids, including potential its mobile DNA (eg, such as transposons) may include genes associated with FQ resistant efflux pump has recently been reported as a mechanism of FQ resistance.<sup>32</sup> This horizontal transfer provides the genetic linkage between resistance to FQ and  $\beta$ -lactam drugs.<sup>32-34</sup> 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.<sup>32</sup>

In contrast, amoxicillin treatment was associate with non-MDR in fecal *E. coli* in this study. The predominant mechanism of amoxicillin resistance in Gram-negative bacteria reflects production of  $\beta$ -lactamases that might be either chromosomally encoded or transmitted by plasmids.<sup>34-36</sup> However, high-level resistance to sulfamethoxazole/trimethoprim, but no 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.<sup>37</sup> Acquisition of resistance enzymes horizontally causes high-level resistance to either sulfonamides or trimethoprim, or both, supporting our findings in this study.

## CONCLUSION

Either amoxicillin or enrofloxacin administered at recommended dosing regimens is associated with rapid development of high level antimicrobial resistance to that drug by the majority of fecal coliform, and particularly *E. coli*. Resistance associated with amoxicillin resolved when therapy is discontinued. In contrast, resistance to enrofloxacin

persisted.

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