Molecular mechanisms of antimicrobial resistance in fecal *Escherichia coli* of healthy dogs after enrofloxacin or amoxicillin administration

Sherine A. Aly, Nipattra Debavalya, Sang-Jin Suh, Omar A. Oryazabal, and Dawn M. Boothe

Abstract: *Escherichia coli* respond to selective pressure of antimicrobial therapy by developing resistance through a variety of mechanisms. The purpose of this study was to characterize the genetic mechanisms of antimicrobial resistance in fecal *E. coli* after the routine use of 2 popular antimicrobials. Fourteen resistant *E. coli* isolates, representing predominant clones that emerged in healthy dogs' feces after treatment with either amoxicillin (11 *E. coli* isolates) or enrofloxacin (3 *E. coli* isolates), were tested for mutations in DNA gyrase (*gyrA* and *gyrB*) and in topoisomerase IV (*parC*) and for the presence of β-lactamases (bla_{TEM}, bla_{SHV}, bla_{PSE-1} and bla_{CTX-M}) and plasmid-mediated quinolone resistance (*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib*, and *qepA*), by polymerase chain reaction. *Escherichia coli* isolates cultured following amoxicillin therapy only expressed single-drug resistance to β-lactams, while the isolates cultured from dogs receiving enrofloxacin therapy expressed multidrug resistance (MDR). The use of RND efflux pump inhibitors increased the susceptibility of the 3 MDR *E. coli* isolates to doxycycline, chloramphenicol, enrofloxacin, and ciprofloxacin, which indicates a role of the efflux pump in the acquisition of the MDR phenotype. Amplification and sequencing of AcrAB efflux pump regulators (*soxR*, *soxS*, *marR*, and *acrR*) revealed only the presence of a single mutation in *soxS* in the 3 MDR isolates.

Key words: Escherichia coli, β-lactams, fluoroquinolone, antimicrobial resistance.

Résumé : *Escherichia coli* répond à la pression sélective de la thérapie antimicrobienne en développant une résistance par l'intermédiaire d'une variété de mécanismes. Le but de cette étude était de caractériser les mécanismes génétiques de résistance antimicrobienne chez *E. coli* fécal après une utilisation de routine de 2 antimicrobiens populaires. La présence de mutations dans les gènes de l'ADN gyrase (*gyrA* and *gyrB*) et de la topoisomérase IV (*parC*), la présence de β-lactamases (bla_{TEM}, bla_{SHV}, bla_{PSE-1} et bla_{CTX-M}) et la résistance à la quinolone conférée par plasmides (*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib* et *qepA*) ont été testées par réaction en chaine par polymérase chez quatorze isolats résistants d'*E. coli*, représentant des clones prédominants qui émergeaient des fèces de chiens en bonne santé après un traitement à l'amoxilline (11 isolats d'*E. coli*) ou à l'enrofloxacine (3 isolats d'*E. coli*). Les isolats d'*E. coli* cultivés après une thérapie à l'amoxilline seulement exprimaient une résistance simple aux β-lactames alors que les isolats cultivés à partir de chiens traités à l'enrofloxacine exprimaient une résistance multiple aux médicaments (MDR). L'utilisation d'inhibiteurs de la pompe d'efflux RND augmentait la susceptibilité de 3 isolats d'*E. coli* MDR à la doxycycline, au chloramphénicol, à l'enrofloxacine et à la ciprofloxacine, ce qui indique que la pompe d'efflux joue un rôle dans l'acquisition du phénotype MDR. L'amplification et le séquençage des régulateurs de la pompe d'efflux (*soxR*, *soxS*, *marR* et *acrR*) ne révélaient la présence que d'une seule mutation à l'intérieur de *soxC* chez les 3 isolats MDR.

Mots-clés : Escherichia coli, β-lactames, fluoroquinolone, résistance antimicrobienne.

[Traduit par la Rédaction]

Received 7 May 2012. Revision received 22 August 2012. Accepted 23 August 2012. Published at www.nrcresearchpress.com/cjm on 5 November 2012.

S.A. Aly.* Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt.
N. Debavalya† and D.M. Boothe. Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, 109 Greene Hall, AL 36849, USA.
S.-J. Suh. Department of Biological Sciences, Auburn University, Auburn, AL 36849, USA.
O.A. Oryazabal. Department of Biological Sciences, Alabama State University, Montgomery, AL 36101, USA.

Corresponding author: Dawn M. Boothe (e-mail: boothdm@auburn.edu).

*Present address: Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA.
†Present address: Department of Veterinary Pharmacology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

Introduction

Selective pressures resulting from previously administered antimicrobial therapy play important roles in the emergence of antimicrobial resistance. Use of antimicrobials selects for resistance not only in pathogenic bacteria but also in commensal bacteria (Sørum and Sunde 2001; Moyaert et al. 2006). Amoxicillin, enrofloxacin (dogs), or ciprofloxacin (humans and dogs) are among the most commonly used antimicrobials in veterinary and human medicine. Amoxicillin is a semisynthetic β-lactam, while enrofloxacin is a synthetic fluoroquinolone (FQ) (Prescott 2006; Boothe 2012). Resistance to both amoxicillin and FQs in companion animals and humans has been reported in several countries (Costa et al. 2004; Shaheen et al. 2009).

As with other FQs, bacterial resistance to enrofloxacin is principally mediated by mutations in genes encoding for the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC*) (Hooper 1999). However, plasmid-mediated quinolone resistance (PMQR) has become an important emerging issue (Martínez-Martínez et al. 1998). Moreover, increases in minimum inhibitory concentration (MIC) to FQ can also be caused by mutations in genes that maintain microbial impenetrability of compounds, including quinolones. Mechanisms include increasing substrate efflux from the cell or decreasing influx through downregulation of outer membrane proteins (Jellen-Ritter and Kern 2001).

The polyspecificity of bacterial efflux pumps can simultaneously confer resistance to different classes of antibiotics and cause the microorganism to become multidrug resistant (MDR) (Poole 2000). AcrAB is the main efflux pump for *Escherichia coli* associated with FQ resistance. A recent study suggested that *acrAB* overexpression is strongly correlated with FQ-associated MDR (Swick et al. 2011). The expression of *acrAB* is subject to multiple levels of regulation, locally by AcrR (Ma et al. 1996) and globally by SoxS and MarA (Hirakawa et al. 2008).

In contrast to FQ, amoxicillin resistance is most frequently mediated by β-lactamases (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$), which inactivate the compound by hydrolyzing their lactam ring. Resistance to β-lactams tends to be horizontally transmitted via transferrable genetic materials, such as plasmids, integrons, or conjugative transposons (Jacoby and Bush 2005; Li et al. 2007; Cha et al. 2008).

The purpose of this study was to characterize the genetic mechanisms of resistance to either enrofloxacin or amoxicillin in fecal *E. coli* of healthy dogs after the oral administration of such antimicrobials at recommended doses.

Materials and methods

Fecal *E. coli* isolates

The experimental *E. coli* isolates used in this study were collected from feces of healthy, normal dogs with no previous history of antibiotic intake. These dogs were treated orally with amoxicillin (10 mg/kg bid; $n = 8$), enrofloxacin (5 mg/kg once daily; $n = 8$), or no drug (control = 8) for 7 days. Isolates used in this study were drawn from a larger study investigating the impact of routine antimicrobial therapy on the emergence of fecal *E. coli* resistance (Debavalya 2009). Briefly, *E. coli* isolates were collected from feces of healthy, normal dogs that were treated orally with amoxicillin ($n = 8$), enrofloxacin ($n = 8$), or no drug ($n = 8$) for 7 days. Fecal samples were collected digitally from each dog at baseline, after 7 days of treatment and 21–28 days post-treatment. Samples were collected at 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 of fecal sample in 0.9% sodium chloride solution. Once the appropriate dilution was identified, 0.1 mL was plated onto the surface CHROMagar *E. coli* agar plates (CHROMagar, Paris, France, Becton Dickinson) containing no drug, amoxicillin, or enrofloxacin at a concentration equivalent to 1 tube dilution below the resistant breakpoint MIC (MIC_{BP}) as determined by the Clinical Laboratory Standards Institute (CLSI) (2 μg/mL agar for enrofloxacin). CHROMagar *E. coli* agar is a culture medium used for the detection and enumeration of *E. coli* based on the presence of β-glucuronidase enzyme. With CHROMagar *E. coli*, colonies of *E. coli* develop with an intense blue colour, while other Gram-negative bacteria appear colorless and Gram-positive bacteria are inhibited (Alonso et al. 1996). Plates were incubated at 37 °C for 18–24 h (CLSI 2008). Isolates were confirmed to be *E. coli* by screening with IMViC tests (indole, methyl red, Voges–Proskauer, and citrate) (Remel/ Thermo Fisher Scientific, Lenexa, Kansas). From each of the 3 time points for each group of dogs, 10 resistant *E. coli* colonies (for a total of 180 isolates) were randomly selected and their antimicrobial susceptibility and pulsed-field gel electrophoresis (PFGE) DNA fingerprints were determined. The animal use was conducted according to the guidelines of the *Guide to the Care and Use of Experimental Animals.* The experiment followed the ethical principles of Auburn University and was approved by the Institutional Animal Care and Use Committee of the University.

Presence of extended-spectrum β-lactamases

The *E. coli* isolates were tested for extended-spectrum β-lactamase (ESBL) production using microdilution-based Sensititre (TREK diagnostic systems, Cleveland, Ohio) with ESBL Confirmatory MIC plates (ESB1F). Presence of ESBL was based on susceptibility to 9 narrow-spectrum 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) and 2 carbapenems (meropenem and imipenem), according to the manufacturer's instructions. The susceptibility status of each drug was based on comparisons of the MIC for that drug to that of the CLSI antimicrobial susceptibility standards as delineated in M100-S18 (CLSI 2008). When the addition of the β-lactamase inhibitor (clavulanic acid) resulted in ≥3-fold serial dilution decrease in MIC of either antibiotic, the isolate was confirmed as an ESBL producer.

Impact of efflux pump inhibitor (EPI) on MIC

Susceptibilities to 8 antimicrobial drugs (amoxicillin, amoxicillin–clavulanic acid, cefpodoxime, amikacin, ciprofloxacin, enrofloxacin, doxycycline, and chloramphenicol) were studied by the broth microdilution method in the presence or absence of 20 mg/L concentration of Phe-Arg-β-naphthylamide (PAβN), in accordance with the CLSI guidelines (CLSI 2008). Custom microtitre plates containing selected antimicrobials at increasing concentrations were purchased from Sensititre Vizion System (TREK diagnostic systems, Cleveland, Ohio, USA). For qual-

Table 1. Oligonucleotides used for PCR and DNA sequencing.

Primer	Nucleotide sequence	Target size (bp)	Reference
GyrA-F	5′-TGCCAGATGTCCGAGAT	269	This study
GyrA-R	5′-GTATAACGCATTGCCGC		
GyrB-F	5′-CAGACTGCCAGGAACGCGAT	203	This study
GyrB-F	5′-AGCCAAGCGCGGTGATAAGC		
ParC-F	5′-TATGCGATGTCTGAACTGGG	264	This study
ParC-R	5′-GCTCAATAGCAGCTCGGAAT		
TEM-F	5′-GCTCAGTATTGCCCGCTCCAC	1013	This study
TEM-R	5′-ACTACGATACGGGAGGGCTTACCA		
SHV-F	5′-TATTCGCCTGTGTATTATCTCC	855	This study
SHV-R	5′-TTTTAGCGTTGCCAGTGC		
CTX-M F	5′-TTTGCGATGTGCAGTACCAGTAA	523	Edelstein et al. 2003
CTX-M R	5′-CGATATCGTTGGTGGTGCCATA		
SoxR-F	5′-TGCGGAACATTCGTTGCAAGTACC	825	This study
SoxR-R	5′-AAAGCATCAACACCAACCGGAACC		
SoxS-F	5′-TTGTTGAAACGCTGACCAC	867	This study
SoxS-R	5′-CCAGCGGAATGCCAATA		
MarR-F	5′-CATTGGTGGTTGTTATCCTGTGTA	899	This study
MarR-R	5′-TATCGGCTCGTTACTTTCCTTC		
AcrR-F	5′-GAACCTGAAGAACGACCTGA	1172	This study
AcrR-R	5′-CATCAGAACGACCGCACGAG		
QnrA-F	5′-ATTTCTCACGCCAGGATTTG	518	Robicsek et al. 2006
QnrA-R	5′-GATCGGCAAAGGTTAGGTCA		
QnrB-F	5′-GATCGTGAAAGCCAGAAAGG	469	Robicsek et al. 2006
QnrB-R	5′-ACGATGCCTGGTAGTTGTCC		
QnrS-F	5′-CGAGATCAATTTACGGGGAATA	417	Gay et al. 2006
QnrS-R	5′-AACAAGCTGAAGCGCCTG		
Aac(6′)-F	5′-TTGCGATGCTCTATGAGTGGCTA	482	Park et al. 2006
Aac(6′)-R	5′-CTCGAATGCCTGGCGTGTTT		
QepA-F	5′-GCAGGTCCAGCAGCGGGTAG	218	Liu et al. 2008
QepA-R	5′-CTTCCTGCCCGAGTATCGTG		

ity control purposes *E. coli* ATCC 25922 (American Tissue Cell Culture, Manassas, Virginia, USA) was used.

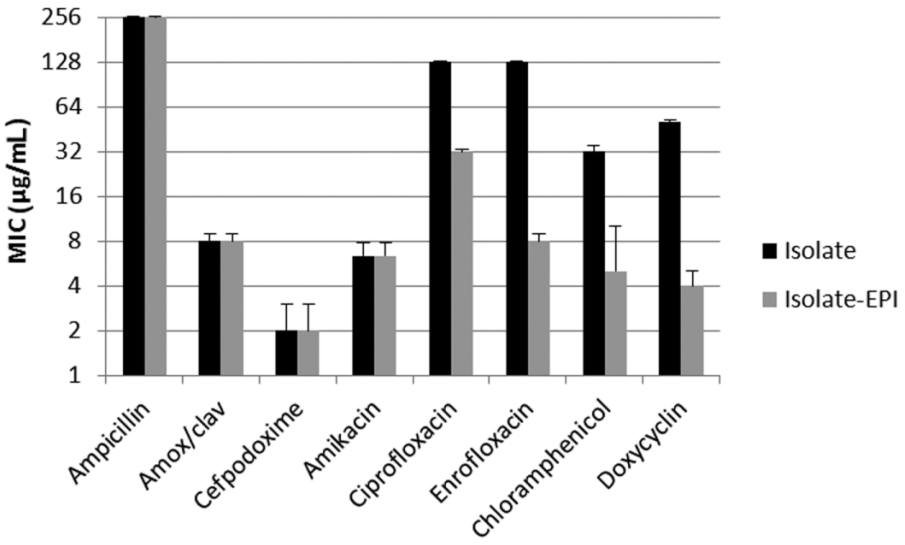
Identification of putative mechanisms of resistance

The *E. coli* isolates were tested for mutations in genes coding for DNA gyrase (*gyrA* and *gyrB*), topoisomerase IV (*parC*), and transcriptional regulators of AcrAB efflux pump (*soxR*, *soxS*, *marR*, and *acrR*). The presence of plasmid-encoded *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, and *qepA* was screened by polymerase chain reaction (PCR). *Escherichia coli* J53 strains containing pMG252, pMG298, pMG306, and pMG298 were used as *qnrA*-, *qnrB*-, *qnrS*-, and *aac(60)-Ib-cr*-positive controls, respectively. *Escherichia coli* J7261205 (pSTVqepA) was included as a positive control for *qepA* (Cattoir et al. 2007; Robicsek et al. 2006; Yamane et al. 2008). A multiplex PCR was used to screen for β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{PSE-1}, and bla_{CTX-M}). The ATCC reference strain W3110 (wild-type *E. coli*) was used as the control isolate. PCR amplification of these genes was performed in a Multigene Gradient Thermocycler PCR System (Labnet International Inc., Edison, New Jersey, USA). The oligonucleotide primers (Table 1) were designed using Lasergene software package (DNASTAR, Madison, Missouri, USA), based on the published sequences of the genes (GenBank accession numbers) on the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov). Following amplification, PCR products were visualized in 1% agarose gel with ethidium bromide and analyzed under UV light. For detection of mutation, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, California, USA) and sequenced at the DNA Core Facility of the Massachusetts General Hospital (Cambridge, Massachusetts, USA). The *soxS* sequence was deposited in GenBank under the accession No. GU 994539.

Conjugative transfer of resistance genes

To determine the transmissibility of the resistance determinants, conjugation was performed on the 3 MDR *E. coli* isolates. Horizontal transfer of antimicrobial resistance genes was investigated in all isolates with the conjugation method modified from Miller (1972). Briefly, all isolates were grown on Luria–Bertani (LB) broth to logarithmic growth phase (OD_{600}, 0.5–0.6). Conjugations were performed by mixing equal amounts of cultures of *E. coli* isolates and 2 different *E. coli* recipients: GN 3201 containing Tn*5* (Tn*5*::Kan^r) and DH5α (*recA*$^-$, Nal^r). CAG 5051 (Hfr, *nadA-57*::Tn*10*) *E. coli* containing Tn*10* with tetracycline-resistant mutant was used as a positive control. The suspensions were spotted on LB plates and incubated overnight at 37 °C to allow conjugation. Transconjugants were selected by plating the mated mixture

Fig. 1. The average minimum inhibitory concentration (MIC) of the multidrug-resistant *Escherichia coli* isolates ($n = 3$) before and after the addition of the efflux pump inhibitor (EPI) Phe-Arg-β-naphthylamide (20 mg/L).



on LB agar plates containing nalidixic acid, nalidixic acid + enrofloxacin, and enrofloxacin at the drug concentration one tube below the MIC_{BP} (50 μg/mL kanamycin 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 of the plates were incubated at 37 °C for 24–48 h.

Results

Resistant *E. coli* isolates

No fecal *E. coli* isolates cultured in baseline samples from any of the 3 groups expressed resistance to either amoxicillin or enrofloxacin. However, within 7 days of therapy, 100% of fecal *E. coli* isolates cultured from dogs receiving amoxicillin therapy were found resistant against this drug. All the tested isolates were single-drug resistant (SDR), expressing a high level of resistance to β-lactams ($MIC_{90} \geq 256$) only. Likewise, within 7 days of therapy, 100% of *E. coli* isolates cultured from dogs receiving FQ therapy expressed a high level of resistance to enrofloxacin ($MIC_{90} \geq 32$), but these isolates were all found expressing MDR, as characterized by resistance to 6 other drug classes. Thirteen PFGE DNA fingerprinting classes and 7 antibiotic-resistant phenotypes were described among the 180 isolates. From these 2 groups of dogs, a total of 14 *E. coli* isolates representing each PFGE class within each antibiotic-resistant phenotype were further characterized: 11 SDR *E. coli* isolates from amoxicillin-treated dogs and 3 MDR *E. coli* isolates from FQ-treated dogs. ESBL production was detected in 5 SDR *E. coli* isolates, with ≥3-fold dilution decreases in MICs of the third generation cephalosporins in the presence of clavulanic acid, compared with MICs of the single drugs. In contrast, no ESBL was detected in the other 6 SDR *E. coli* isolates or the 3 MDR *E. coli* isolates (Table 2).

Table 2. *Escherichia coli* isolates tested in this study.

Isolate	Treatment	Phenotype[a]	MDR	ESBL	Pulsotype
11	Amoxicillin	ABPRL	–	–	2
24	Amoxicillin	ABPRL	–	–	3
31	Amoxicillin	ABPRL	–	–	4
32	Amoxicillin	ABPRL	–	–	5
36	Amoxicillin	ABPRL	–	–	6
38	Amoxicillin	ABPRL	–	–	7
161	Amoxicillin	ABPRLC	–	+	8
164	Amoxicillin	ABPRLC	–	+	9
171	Amoxicillin	ABPRLC	–	+	10
123	Amoxicillin	ABPRLC	–	+	1
132	Amoxicillin	ABPRLC	–	+	11
61	Enrofloxacin	ABPRLFET	+	–	12
71	Enrofloxacin	ABPRLFETH	+	–	12
81	Enrofloxacin	ABPRLFET	+	–	13

Note: MDR, multidrug resistant; ESBL, extended-spectrum β-lactamase.
[a]A, ampicillin; B, carbenicillin; P, piperacillin; R, ticarcillin; L, cephalothin; C, ceftiofur; F, ciprofloxacin; E, enrofloxacin; T, tetracycline; H, chloramphenicol.

Changes in antimicrobial susceptibility of *E. coli* isolates with EPI

In the 3 MDR *E. coli* isolates from dogs treated with enrofloxacin, the MIC values changes were profound in the presence of EPI for selected antimicrobials. The results show that the use of a 20 mg/L concentration of PAβN decreased the MIC of ciprofloxacin (2-fold dilution), enrofloxacin (4-fold dilution), doxycycline (from 3- to 4-fold serial dilution), and chloramphenicol (from 2- to 3-fold serial dilution) in the 3 tested MDR *E. coli* isolates (Fig. 1). No change in the MIC has been detected in the 11 SDR *E. coli* isolates from amoxicillin-treated dogs after the use of EPI.

Detection of β-lactamase genes

bla$_{TEM}$ was detected in the 6 non-ESBL-producing SDR *E. coli* and the 3 MDR *E. coli* isolates but not in the 5 ESBL-producing SDR isolates. Conversely, *bla*$_{CTX-M}$ was present only in the 5 ESBL SDR *E. coli* isolates. No *bla*$_{SHV}$ or *bla*$_{PSE-1}$ was detected in any of the resistant or control isolates (Table 3).

Mutations in quinolone resistance determining region genes

The presence of 2 mutations in *gyrA* at codons 83 and 87 and 2 mutations of *parC* at codons 80 and 84 were detected in the 3 MDR *E. coli* isolates. No mutations in *gyrB* were found in any of the isolates. For mutations in *gyrA*, the mutation at codon 83 was a C→T transition in the codon TCG, resulting in the substitution of leucine for serine, while the mutation at codon 87 was a G→A transition of codon GAC, leading to the substitution of asparagine for aspartate. For *parC*, a mutation at codon 80 was 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 an A→C transversion of codon GAA (Table 3). No mutations in quinolone resistance determining region genes were detected in the SDR *E. coli* isolates and the wild-type *E. coli* strain W3110.

Mutations in efflux pump regulators

Overexpression of the AcrAB efflux pump can result from mutations in one of several *acrAB* regulator genes, including *soxR*, which renders the protein active; *soxS* promoter, which initiates transcription of the *soxS* gene constitutively; *marR*, which suppresses MarA activity with the subsequent release of the inhibition exerted on *marA*; or *acrR*, which alleviates the suppression exerted on *acrAB* (Martin and Rosner 2002). To address these possibilities we amplified and sequenced the genes that encode for the 4 regulators: *soxR*, *soxS*, *marR*, and *acrR*.

The 3 MDR *E. coli* isolates had only a single mis-sense mutation in *soxS* in codon 12 with G→T transversion in codon GCA, leading to substitution of alanine with serine. No mutations were detected in *soxS*, *soxR*, *marR*, and *acrR* in any of the 11 SDR *E. coli* isolates or the wild-type strain (Table 3).

Detection of PMQR

Only *qnrB* was detected in one of the MDR *E. coli* isolates. No plasmids were detected in other isolates. This suggests that PMQR did not play a role in FQ resistance in these isolates (Table 3).

Conjugative transfer of resistance genes

In the 5 ESBL-producing *E. coli*, β-lactam resistance was found to be transferred horizontally via conjugation. In contrast, co-transfer of the resistance traits via conjugation was not observed in any of the 6 non-ESBL-producing SDR *E. coli* or the 3 MDR *E. coli* isolates. This suggested that dissemination of resistance was not due to plasmid conjugation in these isolates.

Discussion

Emergence of MDR *E. coli* in response to routine antimicrobial therapy is becoming increasingly problematic in veterinary medicine (Ahmed et al. 2005). The dogs studied were

Table 3. Mutations and amino acid changes detected in single-drug-resistant and multidrug-resistant *Escherichia coli* isolates.

			gyrA		*parC*										β-Lactamases	
Isolate	Phenotype[a]	MDR	S83L	D87N	S80I	E84A	*gyrB*	*soxR*	*soxS*	*marR*	*acrR*	*qnrA*	*qnrB*	*qnrS*	*aac(6′)-Ib-cr*	*qepA*	*bla*$_{TEM}$	*bla*$_{CTX-M}$
501	Wild type	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
24	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
31	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
32	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
36	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
38	ABPRL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
161	ABPRLC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
164	ABPRLC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
171	ABPRLC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
123	ABPRLC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
132	ABPRLC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
61	ABPRLFET	+	+	+	+	+	–	–	A12S	–	–	–	+	–	–	–	+	–
71	ABPRLFETH	+	+	+	+	+	–	–	A12S	–	–	–	–	–	–	–	+	–
81	ABPRLFET	+	+	+	+	+	–	–	A12S	–	–	–	–	–	–	–	+	–

Note: MDR, multidrug resistant.
[a]A, ampicillin; B, carbenicillin; P, piperacillin; R, ticarcillin; L, cephalothin; C, ceftiofur; F, ciprofloxacin; E, enrofloxacin; T, tetracycline; H, chloramphenicol.

healthy, with no previous history of antimicrobial administration. Prior to treatment, the fecal *E. coli* population was predominantly susceptible to either treatment drug, but within 7 days of therapy with either drug at doses recommended for therapeutic intervention, the *E. coli* population was transformed to one predominated by isolates expressing high-level resistance to the treatment drug. Further, whereas amoxicillin therapy was associated with the emergence of SDR fecal *E. coli*, enrofloxacin therapy was associated with the emergence of MDR fecal *E. coli* isolates.

Five of the SDR *E. coli* isolates detected after amoxicillin therapy were associated with ESBL production. Previous studies have demonstrated that β-lactam resistance in ESBL-producing *E. coli* isolates is caused by plasmid-mediated CTX-M β-lactamases transferred horizontally during conjugation (Bonnet 2004). In contrast, β-lactam resistance in either non-ESBL-producing SDR or MDR *E. coli* isolates is more likely to be mediated by nontransferrable TEM β-lactamase enzymes carried by transposons, which are then integrated into the chromosomal DNA (Poirel et al. 2008).

De novo quinolone resistance often develops in a gradual, stepwise manner, usually from the accumulation of mutations that individually lower susceptibility by modest increments (Drlica 2003). Our data demonstrated that the presence of 2 mutations in *gyrA* and 2 mutations in *parC* is associated with a high level of resistance to enrofloxacin (MIC $\geq$ 256) in the resistant *E. coli* isolates, supporting previously published data (Webber and Piddock 2001; Liu et al. 2012). These *E. coli* isolates contained the amino acid substitution of alanine for glutamate at codon 84 of *parC*, which is different than the previously reported mutations in *E. coli*. However, amino acid substitution of alanine for glutamate at codon 84 of *parC* has been previously reported in *Enterococcus fecalis* (Hooper 1999).

The AcrAB MDR efflux pump overexpression has been shown to be associated with high-level FQ resistance in *E. coli* (Webber and Piddock 2001; Wang et al. 2003; Liu et al. 2012) and has been suggested to be strongly correlated with the MDR associated with FQ resistance (Swick et al. 2011; Liu et al. 2012). Susceptibility testing of the 3 MDR *E. coli* isolates in the presence of the RND EPI (PAβN) revealed a decrease in resistance to 4 antimicrobial drugs with the magnitude of resistance ranging from 2- to 4-fold (Fig. 1). These data suggest a role for the AcrAB efflux pump in the MDR phenotype in isolates exposed to therapeutic doses of enrofloxacin. Increased activity of the AcrAB efflux pump can result from overexpression of *soxS* (caused by mutation in *soxR*) or *marA* (due to mutation in *marR*) or from mutations in *acrR* that inactivate the suppressor protein (Wang et al. 2003). In this study, only a single mis-sense mutation in *soxS* was found that changed alanine to serine at position 12. However, there is no information is available regarding the effect of this *soxS* mutation on the AcrAB pump expression.

A debate that this study cannot answer is whether or not antimicrobial therapy induced isolates to acquire resistance through genetic or other changes or if the antimicrobial therapy simply exerted selection pressure, killing all but a minority population of resistant microbes that subsequently emerged as the predominant population. It is likely that both occurred; the drug induced a new minority population of resistance microbes, which subsequently emerged as the predominant population, filling the void left by those isolates killed by the drug. That induction occurred at some level is supported by the emergence of MDR isolates in response to enrofloxacin (but not amoxicillin), with isolates being characterized by both mutations in topoisomerases and increased efflux pump activity. Yet, because our techniques may not have been sensitive enough to detect the presence of a minority population containing these characteristics among a large fecal *E. coli* population, we cannot verify the mechanism by which these resistant populations emerged. However, regardless of the mechanism, what this study demonstrates is the rapid and complete transition of a predominantly susceptible population of *E. coli* to one that expressed resistance to the treatment drug; further, that the resistance that emerged with the fluorinated quinolone therapy was high level and multidrug.

Although this report was based on antimicrobial drug treatment under controlled, experimental conditions, the conditions are typical of those associated with actual clinical settings. Thus, our data suggest the rapid emergence of antimicrobial-resistant mutants in clinical veterinary settings that can contribute to dissemination of drug-resistant commensal bacteria.

Acknowledgements

This work was supported by an Interdisciplinary Equipment and Research Award (2009) and the Auburn University Intramural Grants Program (AU-IGP 2010). We would like to thank NSF-EPSCoR for grant NSF-ESP 0814103 awarded to the Cellular and Molecular Biosciences at Auburn University and the Auburn University Detection and Food Safety Peaks of Excellence program for grant USDA 2009 G00004608 to S.-J. Suh. We would like to acknowledge Ms. Terri Hathcock for her microbiological support.

References

Ahmed, A.M., Miyoshi, S., Shinoda, S., and Shimamoto, T. 2005. Molecular characterization of a multidrug-resistant strain of enteroinvasive *Escherichia coli* O164 isolated in Japan. J. Med. Microbiol. **54**(3): 273–278. doi:10.1099/jmm.0.45908-0. PMID: 15713611.

Alonso, J.L., Amoros, I., Chong, S., and Garelick, H. 1996. Quantitative determination of *Escherichia coli* in water using CHROMagar® *E. coli.* J. Microbiol. Methods, **25**(3): 309–315. doi:10.1016/0167-7012(96)00002-4.

Bonnet, R. 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. **48**(1): 1–14. doi:10.1128/AAC.48.1.1-14.2004. PMID:14693512.

Boothe, D.M. Antimicrobial drugs. 2012. *In* Small animal clinical pharmacology and therapeutics. *Edited by* D.M. Boothe. Elsevier, Philadelphia, USA. pp. 190–270.

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C.J., and Nordmann, P. 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. **60**(2): 394–397. doi:10.1093/jac/dkm204. PMID:17561500.

Cha, J., Kotra, L.P., and Mobashery, S. 2008. Resistance to β-lactam antibiotics mediated by β-lactamases: structure, mechanism, and evolution. *In* Bacterial resistance to antimicrobials. *Edited by* R.G. Wax, K. Lewis, A.A. Salyers, and H. Taber. CRC Press, Boca Raton, Fla., USA. pp. 103–132.

Clinical and Laboratory Standards Institute. 2008. Performance standards for antimicrobial susceptibility testing. 16th Informational

Supplement. CLSI document M100-S18. Clinical and Laboratory Standards Institute, Wayne, Pa., USA.

Costa, D., Poeta, P., Briñas, L., Sáenz, Y., Rodrigues, J., and Torres, C. 2004. Detection of CTX-M-1 and TEM-52 β-lactamases in *Escherichia coli* strains from healthy pets in Portugal. J. Antimicrob. Chemother. **54**(5): 960–961. doi:10.1093/jac/dkh444. PMID:15472002.

Debavalya, N. 2009. Impact of routine antimicrobial therapy on fecal *Escherichia coli* in dogs. Ph.D. thesis, Auburn University, Auburn, Ala., USA.

Drlica, K. 2003. The mutant selection window and antimicrobial resistance. J. Antimicrob. Chemother. **52**(1): 11–17. doi:10.1093/jac/dkg269. PMID:12805267.

Edelstein, M., Pimkin, M., Palagin, I., Edelstein, I., and Stratchounski, L. 2003. Prevalence and molecular epidemiology of CTX-M extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob. Agents Chemother. **47**(12): 3724–3732. doi:10.1128/AAC.47.12.3724-3732.2003. PMID:14638473.

Gay, K., Robicsek, A., Strahilevitz, J., Park, C.H., Jacoby, G., Barrett, T.J., et al. 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin. Infect. Dis. **43**(3): 297–304. doi:10.1086/505397. PMID:16804843.

Hirakawa, H., Takumi-Kobayashi, A., Theisen, U., Hirata, T., Nishino, K., and Yamaguchi, A. 2008. AcrS/EnvR represses expression of the *acrAB* multidrug efflux genes in *Escherichia coli*. J. Bacteriol. **190**(18): 6276–6279. doi:10.1128/JB.00190-08. PMID:18567659.

Hooper, D.C. 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updat. **2**(1): 38–55. doi:10.1054/drup.1998.0068. PMID:11504468.

Jacoby, G., and Bush, K. 2005. β-Lactam resistance in the 21st century. *In* Frontiers in antimicrobial resistance: a tribute to Stuart B. Lavy. *Edited by* D.G. White, M.N. Alekshun, P.F. McDermott, and S.B. Levy. ASM Press, Herndon, Va., USA. pp. 53–65.

Jellen-Ritter, A.S., and Kern, W.V. 2001. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants selected with a fluoroquinolone. Antimicrob. Agents Chemother. **45**(5): 1467–1472. doi:10.1128/AAC.45.5.1467-1472.2001. PMID:11302812.

Li, X.Z., Mehrotra, M., Ghimire, S., and Adewoye, L. 2007. β-Lactam resistance and β-lactamases in bacteria of animal origin. Vet. Microbiol. **121**(3–4): 197–214. doi:10.1016/j.vetmic.2007.01.015. PMID:17306475.

Liu, J.-H., Deng, Y.-T., Zeng, Z.-L., Gao, J.-H., Chen, L., Arakawa, Y., and Chen, Z.-L. 2008. Coprevalence of plasmid-mediated quinolone resistance determinants QepA,Qnr, and AAC(6)-Ib-cr among 16S rRNA methylase RmtB producing *Escherichia coli* isolates from pigs. Antimicrob. Agents Chemother. **52**(8): 2992–2993. doi:10.1128/AAC.01686-07. PMID:18490500.

Liu, X., Boothe, D.M., Thungrat, K., and Aly, Sh. 2012. Mechanisms accounting for fluoroquinolone multidrug resistance *Escherichia coli* isolated from companion animals. Vet. Microbiol. In press. doi:10.1016/j.vetmic.2012.07.019. PMID:22877517.

Ma, D., Alberti, M., Lynch, C., Nikaido, H., and Hearst, J.E. 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19**(1): 101–112. doi:10.1046/j.1365-2958.1996.357881.x. PMID:8821940.

Martin, R.G., and Rosner, J.L. 2002. Genomics of the *marA/soxS/rob* regulon of *Escherichia coli*: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol. Microbiol. **44**(6): 1611–1624. doi:10.1046/j.1365-2958.2002.02985.x. PMID:12067348.

Martínez-Martínez, L., Pascual, A., and Jacoby, G.A. 1998. Quinolone resistance from a transferable plasmid. Lancet, **351**(9105): 797–799. doi:10.1016/S0140-6736(97)07322-4. PMID:9519952.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Woodbury, N.Y., USA.

Moyaert, H., De Graef, E.M., Haesebrouck, F., and Decostere, A. 2006. Acquired antimicrobial resistance in the intestinal microbiota of diverse cat populations. Res. Vet. Sci. **81**(1): 1–7. doi:10.1016/j.rvsc.2005.10.004. PMID:16330058.

Park, C.H., Robicsek, A., Jacoby, G.A., Sahm, D., and Hooper, D.C. 2006. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. **50**(11): 3953–3955. doi:10.1128/AAC.00915-06. PMID:16954321.

Poirel, L., Naas, T., and Nordmann, P. 2008. Genetic support of extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1): 75–81. doi:10.1111/j.1469-0691.2007.01865.x. PMID:18154530.

Poole, K. 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob. Agents Chemother. **44**(9): 2233–2241. doi:10.1128/AAC.44.9.2233-2241.2000. PMID:10952561.

Prescott, J.F. 2006. Beta-lactam antibiotics: penam penicillins, *In* Antimicrobial therapy in veterinary medicine. 4th ed. *Edited by* S. Giguere, J.F. Prescott, J.D. Baggot, R.D. Walker, and P.M. Dowling. Blackwell Publishing, Ames, Iowa. pp. 121–137

Robicsek, A., Strahilevitz, J., Sahm, D.F., Jacoby, G.A., and Hooper, D.C. 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. **50**(8): 2872–2874. doi:10.1128/AAC.01647-05. PMID:16870791.

Shaheen, B.W., Wang, C., Johnson, C.M., Kaltenboeck, B., and Boothe, D.M. 2009. Detection of fluoroquinolone resistance level in clinical canine and feline Escherichia coli pathogens using rapid real-time PCR assay. Vet. Microbiol. **139**(3–4): 379–385. doi:10.1016/j.vetmic.2009.05.016. PMID:19596530.

Sørum, H., and Sunde, M. 2001. Resistance to antibiotics in the normal flora of animals. Vet. Res. **32**(3–4): 227–241. doi:10.1051/vetres:2001121. PMID:11432415.

Swick, M.C., Morgan-Linnell, S.K., Carlson, K.M., and Zechiedrich, L. 2011. Expression of multidrug efflux pump genes *acrAB-TolC*, *mdfA* and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob. Agents Chemother. **55**(2): 921–924. doi:10.1128/AAC.00996-10. PMID:21098250.

Wang, M., Tran, J.H., Jacoby, G.A., Zhang, Y., Wang, F., and Hooper, D.C. 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob. Agents Chemother. **47**(7): 2242–2248. doi:10.1128/AAC.47.7.2242-2248.2003. PMID:12821475.

Webber, M., and Piddock, L.J.V. 2001. Quinolone resistance in *Escherichia coli.* Vet. Res. **32**(3–4): 275–284. doi:10.1051/vetres:2001124. PMID:11432418.

Yamane, K., Wachino, J., Suzuki, S., and Arakawa, Y. 2008. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. Antimicrob. Agents Chemother. **52**(4): 1564–1566. doi:10.1128/AAC.01137-07. PMID:18285488.