Evaluation of the contribution of *gyrA* mutation and efflux pumps to fluoroquinolone and multidrug resistance in pathogenic *Escherichia coli* isolates from dogs and cats

Bashar W. Shaheen, DVM, PhD; Dawn M. Boothe, DVM, PhD; Omar A. Oyarzabal, DVM, PhD; Chengming Wang, DVM, PhD; Calvin M. Johnson, DVM, PhD

Objective—To investigate the contribution of *gyrA* mutation and efflux pumps to fluoroquinolone resistance and multidrug resistance among *Escherichia coli* isolates from dogs and cats.

Sample Population—536 clinical isolates of *E coli*.

Procedures—Minimum inhibitory concentrations (MICs) were determined for enrofloxacin and 6 other drug classes by use of broth microdilution techniques. Real-time PCR assay was used to determine the mutation in *gyrA*; Phe-Arg-β-naphthylamide, an efflux pump inhibitor, was used to examine the contribution of efflux pump overexpression.

Results—The MIC for fluoroquinolones increased in a stepwise fashion and was lowest in the absence of mutations, higher with a single point mutation, and highest with 2 point mutations. Level of resistance in the latter category was high (8 times the breakpoint), but this was associated with expression of the AcrAB efflux pump. Inhibition of the efflux pump resulted in a reduction in the MIC to less than the susceptible breakpoint for isolates with an MIC ≤ 4 mg/L, regardless of the presence of a mutation. The greatest magnitude in MIC decrease (MIC was decreased by a factor of > 67 fold) was for isolates with a single mutation but the greatest absolute decrease in MIC (124 mg/L) was for isolates with 2 mutations. Inhibition of the AcrAB efflux pump in isolates characterized by multidrug resistance decreased the MIC of drugs structurally unrelated to fluoroquinolone.

Conclusions and Clinical Relevance—Fluoroquinolone resistance in *E coli* appeared to be a stepwise phenomenon, with MIC increasing as the number of point mutations in *gyrA* increased, but high-level resistance and multidrug resistance associated with fluoroquinolone resistance reflected overexpression of the AcrAB efflux pump. (*Am J Vet Res* 2011;72:25–32)

Escherichia coli is a major cause of urinary tract infections and has an important role in infections of other tissues, particularly in dogs.[1,2] Fluoroquinolones are among the drugs most commonly used to treat infections caused by *E coli* in dogs and cats. This frequent use may have contributed to the substantial increase in *E coli* resistance to fluoroquinolone that has emerged in the past decade.[3–5] Because fluoroquinolones are used by veterinarians and physicians as first-line treatment in the United States and Europe[3,6] and *E coli* is a common cause of urinary tract and other infections in humans, its resistance is both a medical concern (therapeutic failure in the veterinary patient) and a public health concern (transmission of resistant *E coli* from pets to people and back to pets).[4,7,8] Furthermore, fluoroquinolone resistance in *E coli* is often associated with MDR, as has been found in *E coli* isolates collected from dogs and cats with clinical infections.[3,8,a]

Abbreviations

EPI	Efflux pump inhibitor
FRET	Fluorescence resonance energy transfer
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MPC	Mutation prevention concentration
PAβN	Phe-Arg-β-naphthylamide
QRDR	Quinolone resistance–determining region
RND	Resistance-nodulation-division

Received June 20, 2009.
Accepted November 9, 2009.
From the Departments of Anatomy, Physiology and Pharmacology (Shaheen, Boothe) and Pathobiology (Wang, Johnson), College of Veterinary Medicine, and Department of Poultry Science, College of Agriculture (Oyarzabal), Auburn University, Auburn, AL 36849. Dr. Shaheen's present address is Division of Microbiology, National Center for Toxicological Research, US FDA, Jefferson, AR 72079. Dr. Oyarzabal's present address is Department of Biological Sciences, College of Arts and Sciences, Alabama State University, Montgomery, AL 36104. Dr. Wang's present address is Department of Pathobiology, School of Veterinary Medicine, Ross University, Basseterre, St Kitts, West Indies.
Supported in part by grant D07-MS 006 from Morris Animal Foundation.
Address correspondence to Dr. Boothe (boothdm@auburn.edu).

The most common mechanism of resistance to quinolones in *E coli* is mutation in the genes that encode the subunits of the quinolone target topoisomerases, specifically DNA gyrase (topoisomerase II, in *gyrA*), and topoisomerase IV (in *parC*).[9,10] These mutations are located in the QRDR of *gyrA* and its homologous region of *parC*.[10,11] Mutations in *gyrB* and *parE* are less important and rarely contribute to quinolone resistance.[12] Double mutations in *gyrA* are generally required for high-level resistance,[13,14] whereas mutations in *parC* are less frequent and are associated with lower-level resistance.[9,11] In addition to mutations in the QRDR, resistance to quinolones is now known to be mediated by transferable resistance genes (*qnrA*, *B*, *C*, and *S*), enzymatic modification (*aac*[*6'*]-*Ib-cr*), or specific efflux pumps (*qepA*).[15–17] Although these mechanisms confer only low-level resistance, their presence enhances the frequency of selection of chromosomal mutants upon exposure to fluoroquinolones.[15–17]

Although a common cause of fluoroquinolone resistance, mutations in *gyrA* and *parC* will not directly cause MDR. Mechanisms of resistance that involve other classes of antimicrobials must be involved in the emergence of MDR. Among the other mechanisms by which gram-negative bacteria become resistant to fluoroquinolone is decreased intracellular drug concentration caused by increased efflux pump activities.[9,12] Among the different efflux transport proteins, the AcrAB-TolC system is the most active in *E coli*. This system belongs to the RND family of transporters. This system transports a broad range of substrates, including drugs, endogenous substrates, and toxins.[18] Among the drug substrates for this system are chloramphenicol, lipophilic β-lactams, fluoroquinolones, tetracycline, rifampin, novobiocin, fusidic acid, and nalidixic acid.[18,19] It is assumed that the evolutionary role of the MDR efflux pump is to protect bacteria against hostile environments (ie, through the transport of harmful substances out of the bacterial cell). Enteric pathogens (eg, *E coli*) constitutively express efflux pump activities that enable them to survive in environments rich in bile salts and fatty acids, which are substrates for *E coli* AcrAB-TolC.[20] However, overexpression of the AcrAB-TolC system in *E coli* is insufficient to cause resistance to ciprofloxacin, chloramphenicol, tetracycline, and cotrimoxazole.[18,19] It is the combination of a mutation in the QRDR genes with overexpression of an efflux pump that gives rise to high-level resistance to fluoroquinolones.[21] Thus, the induction of AcrAB-TolC efflux pump by efflux pump substrate, such as fluoroquinolones, can cause an increase in resistance to fluoroquinolones and the emergence of MDR phenotypes.[22] The increase of pump expression in the presence of mutations in the QRDR probably explains the high-level resistance associated with MDR.

The AcrAB-TolC efflux pump can be inhibited by a variety of substrates. Efflux pump inhibitors may enhance the activity of some antimicrobials, particularly for gram-negative bacteria, including the Enterobacteriaceae.[10] Among the inhibitors of the AcrAB-TolC system, EPI PAβN reduces resistance caused by the RND efflux systems and can reverse the resistance for antimicrobials that are substrate of the AcrAB-TolC efflux pump.

The association of fluoroquinolone and MDR is generally not addressed in veterinary medicine, especially in companion animals.[3,4,8,23] Studies[9,12] addressing these mechanisms have been largely limited to human strains or isolates of public health concern, such as those collected from food animals. The purpose of the study reported here was to investigate the contribution of *gyrA* mutation and efflux pumps to fluoroquinolone resistance and MDR among *E coli* isolates from dogs and cats.

Materials and Methods

Bacterial isolates and culture conditions—Canine and feline pathogenic *E coli* isolates (n = 536 [395 canine and 141 feline isolates]) were acquired from clinical veterinary microbiology laboratories between May and December 2008. Isolates had been cultured from samples received by the laboratories from veterinary practitioners after collection from dogs or cats with presumed infections. Laboratory personnel submitted to the Clinical Pharmacology Laboratory at Auburn University all *E coli* isolates that were obtained from canine or feline samples during the study period. Isolates were submitted as pure cultures on a trypticase agar slant with overnight shipment at ambient temperature. Upon arrival at the laboratory, the identity of the isolates was further confirmed following incubation on MacConkey agar plates and biochemical testing.[b] Isolates were plated on chromogenic culture media[c] for differentiation and confirmation of the identification of *E coli* and for detection of any misidentified *Enterococcus* spp. Isolates were allocated into 4 geographic regions on the basis of the sample's geographic origin: South, West, Midwest, and Northeast.

Antimicrobial susceptibility testing with and without EPI—Antimicrobial susceptibility testing was performed by use of custom-made microdilution susceptibility plates,[d] according to Clinical and Laboratory Standards Institute guidelines and interpretive standards.[24] A panel of 13 antimicrobial agents was tested (**Appendix 1**). The MIC values were recorded by use of an automated visual read and recording system.[e] For quality control purposes, *E coli* American Type Culture Collection 25922[f] was used. Each isolate was then designated as resistant (R), intermediate (I), and susceptible (S) to enrofloxacin and other drugs by use of Clinical and Laboratory Standards Institute guidelines and interpretive standards.[24] Multidrug resistance was defined as resistance to 2 or more drug classes (the class of β lactams included 3 subgroups: penicillins, cephalosporins, and carbapenems [ie, first, second, and third generations]). For each antimicrobial class, MIC ranges were determined by use of the custom-made microdilution plates.

In preliminary studies, EPI[g] was prepared in 11 mL of Mueller-Hinton broth, then added into custom-made microdilution plates, such that the final concentrations were 10, 25, 50, 100, and 120 mg/L. Inhibition at 100 mg of PAβN/L did not reduce bacterial counts, compared with untreated cultures; accordingly, this concentration of the EPI was used for further studies of the effect of the EPI on overexpression phenotypes among

the clinical isolates. Fifty-six clinical *E coli* isolates were randomly selected such that wide ranges of enrofloxacin MICs were represented. This would allow characterization of the roles of mutation and pump overexpression in fluoroquinolone resistance. Most (43/56) of these clinical isolates had MDR, and 19 fully susceptible isolates were also included. Mutations were determined by use of FRET-PCR assay,[25] and pump overexpression was studied by use of EPI. On the basis of MICs and Clinical and Laboratory Standards Institute guidelines,[24] *E coli* isolates were designated as susceptible (n = 19), intermediate (4), or resistant (33).

An efflux pump–overexpressed phenotype was considered present for any isolate for which the MIC for enrofloxacin decreased at least by a factor of 4 (2 tube dilutions) when the MIC was determined in media containing EPI, compared with media not containing EPI. In addition to enrofloxacin, the impact of EPI on changes in MIC was determined for 13 other drugs.

Detection of *gyrA* gene mutations via FRET-PCR assay—Bacterial DNA for PCR assay was extracted by use of a DNA extraction kit[h] following the manufacturer's instructions. The forward and reverse primers and probes designed specifically for *gyrA* are listed (**Appendix 2**). The length of the amplified product was 310 bp. The copy number of *E coli gyrA* was determined by use of real-time PCR assay in a real-time PCR system.[25,i] The primers and probes were designed by use of statistical software.[j] The execution protocols for stringent PCR assay and melting curve analysis were optimized.[25]

Statistical analysis—The MICs for 50% and 90% of isolates were calculated for each drug and for all 56 isolates subjected to EPI. Furthermore, a cumulative percentage of susceptibility was calculated by use of epidemiological software[k] and was used to compare the MIC of isolates before and after EPI. The odds ratio of resistance in isolates resistant to fluoroquinolone versus isolates susceptible to fluoroquinolone but resistant to other antimicrobial agents associated with single-drug resistance versus MDR was determined. The frequency of resistance to specific antimicrobials in isolates with MDR phenotypes that are resistant to enrofloxacin was investigated. Analyses were performed by use of statistical software.[l] For all comparisons, a value of $P < 0.05$ was considered significant.

Table 1—Antimicrobial resistance pattern (No. [%]) of 72 fluoroquinolone-resistant isolates and 309 susceptible isolates.

Isolate resistance*	FQ-R	Non FQ-R
1 class only	1 (1.4)	207 (67.0)
2 classes	13 (18.1)	63 (20.4)
3 classes	10 (13.9)	29 (9.4)
4 classes	24 (33.3)	6 (1.9)
5 classes	17 (23.6)	4 (1.3)
6 classes	7 (9.7)	0 (0)
MDR phenotype	71 (98.6)	102 (33.0)

*Each of the following represents a unique class for the purpose of study analysis: enrofloxacin; ampicillin, amoxicillin-clavulanic acid, cephalothin, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, meropenem, and ticarcillin-clavulanic acid; doxycycline; chloramphenicol; gentamicin; and trimethoprim-sulfamethoxazole.

FQ-R = Fluoroquinolone-resistant isolates. Non FQ-R = Fluoroquinolone-susceptible isolates resistant to other antimicrobials.

Results

Fluoroquinolone susceptibility patterns—Seventy-one percent (381/536) of the isolates had resistance to 1 or more drugs. Of the 381 isolates expressing a resistant phenotype, 19% (n = 72) included fluoroquinolone resistance. The expression of MDR was greater in isolates with resistance to fluoroquinolones (98.6%), compared with those without resistance to fluoroquinolones (32%; odds ratio, 158.4 [95% confidence interval, 27.3 to 915.4]; **Table 1**). Regarding association of resistance among antimicrobials, isolates resistant to fluoroquinolone were most frequently associated with resistance to β-lactams (89%), followed by doxycycline (72%), gentamicin (41%), chloramphenicol (34%), and trimethoprim-sulfamethoxazole (32%; **Figure 1**).

Target mutations in *gyrA*—Among susceptible isolates (n = 19), most (17) had no *gyrA* mutation in the tested region. However, 2 susceptible isolates with MICs of 0.25 and 0.5 mg/L, respectively, each had a single mutation (ie, S83L and D87G). For intermediate isolates (n = 4; MIC = 1 to 2 mg/L), all isolates had a single *gyrA* mutation (S83L). All resistant isolates (n = 33; MIC ≥ 4 mg/L) expressed either single or double mutations in *gyrA* (ie, S83L, D87N, or both; **Tables 2 and 3**).

Effect of the EPI PAβN—Inhibition of the efflux pump resulted in a reduction in the MIC to less than the susceptible breakpoint (0.5 mg/L) for isolates with an MIC of ≤ 4 mg/L, regardless of the presence of a mutation. Further, EPI decreased the MIC of enrofloxacin in isolates not associated with MDR (Table 2). For those isolates with 2 mutations, EPI resulted in a median decrease in MIC by a factor of 16 (**Figure 2**) and a factor ≥ 32 for isolates with a single mutation. However, the EPI was not able to return isolates with double mutations to a susceptible designation; the median enrofloxacin MIC for those isolates was 4 mg/L. For isolates with low or intermediate resistance (isolates with a single mutation in *gyrA*), the median MIC decreased to

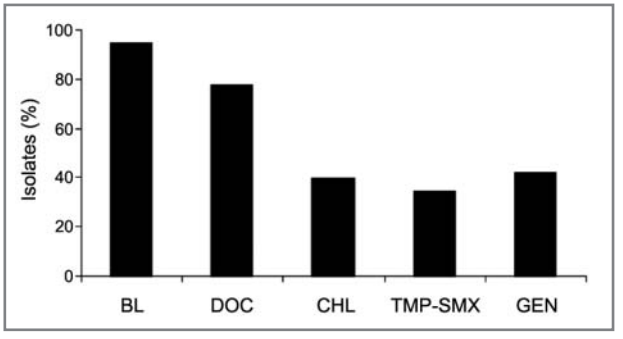

Figure 1—Frequency of resistance to specific antimicrobials in 72 *Escherichia coli* isolates with MDR phenotypes resistant to enrofloxacin. BL = β-lactam class (3 subgroups: penicillins [ampicillin, amoxicillin-clavulanic acid, and ticarcillin-clavulanic acid], carbapenem [meropenem], and cephalosporins [cephalothin, cefoxitin, cefpodoxime, cefotaxime, and ceftazidime]). CHL = Chloramphenicol. DOC = Doxycycline. GEN = Gentamicin. TMP-SMX = Trimethoprim-sulfamethoxazole.

Table 2—Characteristics of clinical isolates of *Escherichia coli* in dogs.

Source	Mutation in QRDR of *gyrA*	Phenotypes of isolates*	Enrofloxacin MIC†		
			Without inhibitor	With inhibitor	Magnitude of MIC decrease
Urine	D87G	MDR[a,c]	0.5	≤ 0.06	≥ 8
Vagina	S83L	MDR[b,f]	0.25	≤ 0.06	≥ 4
Urine		ENR	1	≤ 0.06	≥ 16
		ENR	2	≤ 0.06	≥ 32
		MDR[a–d]	2	≤ 0.06	≥ 32
		MDR[a–d,f]	4	≤ 0.06	≥ 67
	S83L, D87N	MDR[a,b,d–f]	8	1	8
		MDR[a,b,d,e]	16	2	8
		MDR[a,b,d]	16	1	16
		MDR[a–d,f]	32	4	8
		MDR[a,d,f]	32	8	4
Tracheal wash		MDR[a,c–f]	32	4	8
Urine		MDR[a,b,d,e]	64	4	16
		MDR[a–d,f]	64	4	16
		MDR[a,c–f]	64	4	16
		MDR[a–e]	64	4	16
		MDR[a,b,d–f]	64	8	8
Abdominal fluid		MDR[a,b,d–f]	64	4	16
		MDR[b–e]	64	8	8
Fracture		MDR[a,d]	64	8	8
Urine		MDR[a,b,d–f]	64	4	16
		MDR[a,b,d,f]	128	8	16
		MDR[a,b,d]	128	16	8
		MDR[a–f]	128	4	32
		MDR[a,b,d]	128	4	32
		MDR[a–f]	> 128	8	≥ 32
		MDR[a–d,f]	> 128	8	≥ 16
		MDR[a–e]	> 128	8	≥ 16
		MDR[a–f]	> 128	8	≥ 16
		MDR[a–d]	> 128	16	≥ 8
Tracheal wash		MDR[a–f]	> 128	8	≥ 16
Urine	No *gyrA* mutation	NR	0.12	≤ 0.06	≥ 2
		BL	0.12	0.12	0
		BL, MDR[a–c]	0.25	≤ 0.06	≥ 4
		MDR[a,b,f]	0.5	0.12	4
		MDR[a–c,e,f]			
Pleural fluid		BL	0.5	≤ 0.06	≥ 8
Vagina		MDR[a,b,e]	0.5	≤ 0.06	≥ 8
Urine		NR	0.09	≤ 0.06	≥ 1.5
		MDR[a–c]	0.125	≤ 0.06	≥ 2
		MDR[a–c,e]	0.125	≤ 0.06	≥ 2

*Multidrug resistance included the following drug classes: [a]β-lactams, [b]tetracycline, [c]phenicols, [d]fluoroquinolone, [e]potentiated sulfonamides, and [f]aminoglycosides. †Enrofloxacin MIC was determined in the absence and presence of the EPI PAβN (100 mg/L).

BL = β-lactams. ENR = Enrofloxacin. NR = Nonresistant (susceptible) isolates.

Table 3—Characteristics of clinical isolates of *E coli* in cats.

Source	QRDR of *gyrA*	Phenotypes of isolates*	Enrofloxacin MIC†		
			Without inhibitor	With inhibitor	Magnitude of MIC decrease
Urine	S83L	MDR[a–d]	2	≤ 0.06	≥ 32
	S83L, D87N	MDR[a,b,d–f], MDR[a,b,d,f]	32	4	8
		MDR[a,b,d], MDR[a,d–f]	64	8	8
		MDR[a,b,d,f]	64	4	16
		MDR[a–d]	> 128	8	16
		MDR[a,b,d,e]	> 128	16	≥ 8
	No *gyrA* mutation	NR	0.12	≤ 0.06	≥ 2
		BL, NR	0.5	≤ 0.06	≥ 8
		MDR[a–c]	0.09	≤ 0.06	≥ 1.5
Nasal cavity		MDR[a–c,e,f]	0.125	≤ 0.06	≥ 2

See Table 2 for key.

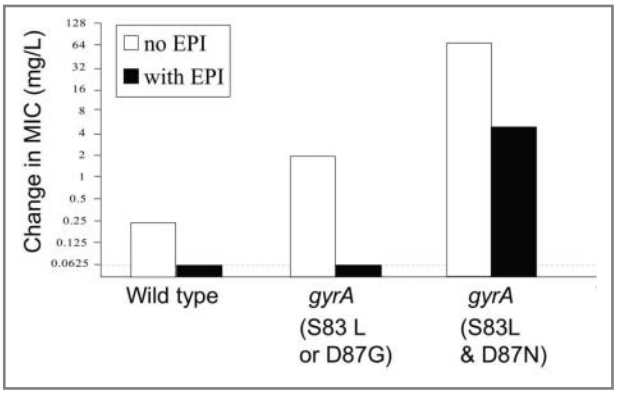

Figure 2—Effect of *gyrA* mutations and EPI on in vitro MICs of enrofloxacin for 56 clinical *E coli* isolates from dogs and cats.

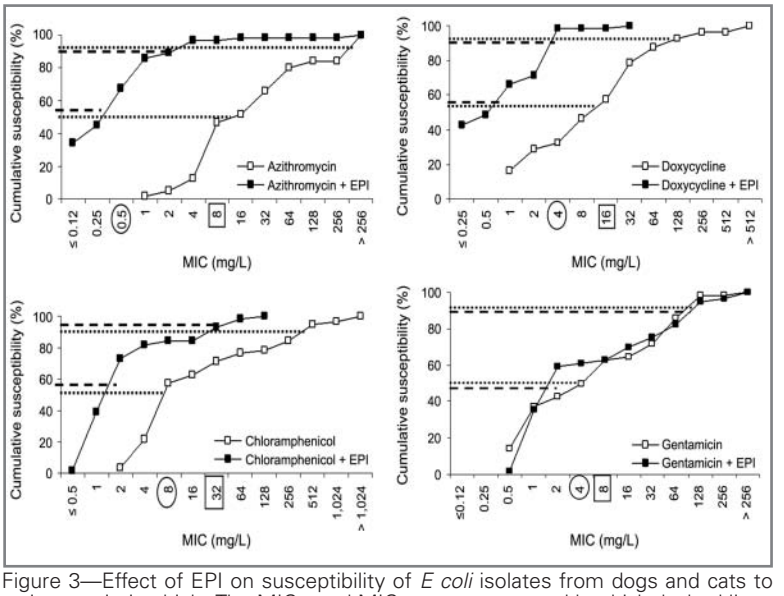

Figure 3—Effect of EPI on susceptibility of *E coli* isolates from dogs and cats to various antimicrobials. The MIC_{90} and MIC_{50} are represented by thick dashed lines for isolates treated with EPI or dotted lines for isolates with no EPI treatment. The MICs in ovals and squares represent the susceptible and resistance breakpoints, respectively.

≤ 0.06 mg/L, which was less than the susceptible breakpoint. However, in terms of magnitude of decrease in MIC, EPI had the greatest impact on enrofloxacin MICs (MIC decrease factor, ≥ 8 to > 67) for the isolates with single mutations in *gyrA* (eg, D87G or S83L; MIC ranging from 0.5 to 4 mg/L; Table 2).

Treatment with EPI also influenced the susceptibility to other selected antimicrobials, including azithromycin, doxycycline, and chloramphenicol. On the basis of changes in the cumulative susceptibility of azithromycin, the percentages of isolates with MICs less than the susceptible and resistant breakpoints were 0% and 46% in the presence of active pumps, respectively, compared with 68% and 96% in the presence of the EPI (**Figure 3**). For doxycycline, the cumulative percentages of susceptible isolates with MICs less than the susceptible and resistant breakpoints were 32% and 57% prior to treatment, respectively, and 98% and 98% after treatment with the EPI. For chloramphenicol, 57% and 71% of the isolates were susceptible without EPI, respectively, compared with 84% and 93% susceptible with EPI. The presence of EPI had no impact on the susceptibility of the isolates to gentamicin.

Discussion

Resistance to fluoroquinolones has increased since their approval in the late 1980s, particularly in the last decade for pathogenic *E coli* and other gram-negative bacteria.[3–5] This resistance limits therapeutic options, may lead to therapeutic failure, appears to be associated with MDR, and is expected to become more prevalent in many veterinary hospitals and clinics.[8] Several surveillance studies have tracked changes in *E coli* susceptibilities to fluoroquinolone and other drug classes over time in humans, but few studies[3,23] have evaluated the mechanisms that account for this increase in resistance in companion animals. The present study evaluated certain mechanisms of resistance among clinical isolates of *E coli*. Most fluoroquinolone-resistant isolates had double mutations in *gyrA*, resulting in a high level (greater than 4 times the resistant breakpoint) of resitance, whereas intermediate to low levels of resistance were found to be associated with a single mutation, which is a finding consistent with previous reports.[14,26] In our study, approximately 82% (9/11) of isolates expressed low MICs (ie, 0.23 to 0.5 mg/L) toward enrofloxacin and had no mutations in *gyrA*. This result indicates that mechanisms other than mutations are important for fluoroquinolone resistance, particularly in isolates lacking *gyrA* mutations. This can be attributed to other resistant determinants such as mutations in regulatory genes and their effect on the constitutive overexpression of efflux pump (ie, AcrAB-TolC system) and porins[18] or PMQR.[15–17] This can be explored further in the future by investigating the contribution of these mechanisms in resistant isolates.

The present study screened for the efficacy of EPI (ie, PAβN) to decrease MICs toward enrofloxacin and other drugs on the basis of its ability to potentiate levofloxacin activity.[18,27] The EPI at low concentration (ie, 25 mg/L) did not effectively decrease MICs to enrofloxacin in *E coli* (data not shown). For enrofloxacin, reduction in the MICs of at least a factor of 4 was observed in almost all of the isolates, with exception of 1 isolate that had an MIC of 0.12 mg/L. These results highlight the importance of basal low-level resistance of gram-negative bacteria to many antimicrobials attributable to a constitutive expression of the efflux pump alone or together with decreased porin expression[9,12,18] The concept of MPC, which is defined as the lowest concentration of antimicrobial to inhibit the emergence of mutants from 10^{10} CFUs,[28] is considered important in preventing the emergence of antimicrobial resistance. This can be of clinical importance especially for isolates that originally did not have mutations in *gyrA* and had low MICs (enrofloxacin MICs of 0.06 mg/L). It is possible that those isolates in vivo could develop high MPC through selection of strains with high-level resistance conferred by mutations in *gyrA*

that enable them to survive initially in the higher concentration of fluoroquinolones. In 1 study,[29] one of the MDR mutants of *Salmonella enterica* serovar Typhimurium (no mutation in *gyrA*) had high MPC:MIC ratios and had low MICs to both ciprofloxacin and enrofloxacin. Further work is needed to determine MPCs of fluoroquinolones in vitro for those isolates and efficacy of different administration regimens in vivo.

Efflux pump inhibitor decreased the MICs for those isolates with a single mutation in *gyrA*, in which the MIC of enrofloxacin decreased from intermediate resistance to susceptible. Interestingly, EPI failed to reduce the MICs for enrofloxacin to less than the susceptible breakpoint (ie, 0.5 mg/L) in the presence of double substitutions in *gyrA*. The presence of an active efflux pump can contribute to further increasing resistance (≥ 8 times) in the presence of a double mutation in *gyrA*.

Although the magnitude in MIC decrease was greatest for isolates with a single mutation, the greatest change in absolute MIC was for isolates with double mutations. For such isolates, EPI reduced from high-level resistance to low-level resistance and from low-level resistance to intermediate resistance. The greatest change in the MIC concentration was 124 mg/L (from 128 to 4 mg/L; Table 2). The therapeutic implication is that for some of these isolates, target MIC can be reached with higher doses. Therefore, it appears that overexpression of the efflux pump alone can have an impact on intracellular drug concentrations, greatly decreasing the concentration such that overexpression of the pump alone could be sufficient to cause resistance to fluoroquinolone and MDR in *E coli*. Evidence to support this result has been documented with *E coli* in which inactivation of the acrAB rendered all strains, including those with target gene mutations, hypersusceptible to fluoroquinolones and other drugs.[30] Similar findings have been reported with other organisms, such as *Pseudomonas aeruginosa*, with deletion of the MexAB-OprM efflux pump[31]; *Salmonella* Typhimurium DT204, with inactivation of *acrB*[32]; and *Campylobacter jejuni*, with *cmeB* deletion.[33]

The present study also determined that EPI reduced the MICs of several antimicrobial drugs; PAβN appeared to have the strongest inhibitory effect on macrolides (ie, azithromycin), drugs not normally considered effective against gram-negative organisms. The EPI also appeared to have no effect on the MIC of an aminoglycoside (ie, gentamicin). This was not surprising because the AcrAB-TolC efflux system in *E coli* has no effect on aminoglycosides,[18] compared with the other efflux system (ie, AcrD), which does affect the efflux of aminoglycosides.[34,35] Furthermore, the EPI did not decrease the MICs of any β-lactams studied. It may be important to note that the PAβN inhibits efflux of some but not all the substrates of RND pumps, including AcrAB-TolC, because the inhibitor-binding site is substrate specific.[36]

Both target gene modification and efflux pump–mediated resistance are underestimated mechanisms by which resistance is mediated in clinical isolates of *E coli* from dogs and cats. A double mutation in *gyrA* was proven to be a major mechanism that contributes substantially to emergence of high-level resistance to fluoroquinolone. A single target-based mutation provided only an intermediate level of resistance to enrofloxacin. Inhibition by EPI of the efflux pump, presumably the AcrAB efflux pump, would substantially affect the MICs for fluoroquinolone and other drug classes. Thus, an alternative treatment could be used to target the efflux system to prevent the emergence of fluoroquinolone-resistant and MDR isolates of *E coli*.

a. Shaheen BW, Boothe DM, Oyarzabal OA, et al. Characterization of clinical *Escherichia coli* isolates expressing multidrug resistance recovered from canine and feline with spontaneous disease (abstr), in *Proceedings*. 26th Annu Meet Am Coll Vet Intern Med Forum 2008;786.
b. Kovacs testing, Remel/Thermo Fisher Scientific, Lenexa, Kan.
c. CHROMagar Orientation, Becton Dickinson, Franklin Lakes, NJ.
d. Micro-dilution susceptibility plates, TREK Diagnostic Systems, Cleveland, Ohio.
e. SENSITITER VIZION system, TREK Diagnostic Systems, Cleveland, Ohio.
f. American Type Culture Collection, Manassas, Va.
g. EPI, Efflux Pump Inhibitor, Phe-Arg-β-naphthylamide (PAβN), Thermofisher, Pittsburgh, Pa.
h. PreMan Ultra Sample Preparation Reagent, Applied Biosystem, Foster City, Calif.
i. LightCycler, Roche Diagnostics, Indianapolis, Ind.
j. Vector NTI 10.1 software, Invitrogen, Carlsbad, Calif.
k. SWIN Epidemiology Module, TREK Diagnostic Systems, Cleveland, Ohio.
l. MINITAB 15 package, Minitab Inc, State College, Pa.

References

1. Chen YM, Wright PJ, Lee CS, et al. Uropathogenic virulence factors in isolates of *Escherichia coli* from clinical cases of canine pyometra and feces of healthy bitches. *Vet Microbiol* 2003;94:57–69.
2. Hagman R, Kühn I. *Escherichia coli* strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. *Vet Microbiol* 2002;84:143–153.
3. Cooke CL, Singer RS, Jang SS, et al. Enrofloxacin resistance in *Escherichia coli* isolated from dogs with urinary tract infections. *J Am Vet Med Assoc* 2002;220:190–192.
4. Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. *J Antimicrob Chemother* 2004;54:321–332.
5. Boothe DM, Boeckh A, Simpson RB, et al. Comparison of pharmacodynamic and pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. *J Vet Intern Med* 2006;20:1297–1306.
6. Goettsch W, van Pelt W, Nagelkerke N, et al. Increasing resistance to fluoroquinolones in *Escherichia coli* from urinary tract infections in the Netherlands. *J Antimicrob Chemother* 2000;46:223–228.
7. Talan DA, Krishnadasan A, Abrahamian FM, et al. Prevalence and risk factor analysis of trimethoprim-sulfamethoxazole- and fluoroquinolone-resistant *Escherichia coli* infection among emergency department patients with pyelonephritis. *Clin Infect Dis* 2008;47:1150–1158.
8. Sanchez S, McCrackin Stevenson MA, Hudson CR, et al. Characterization of multidrug-resistant *Escherichia coli* isolates associated with nosocomial infections in dogs. *J Clin Microbiol* 2002;40:3586–3595.
9. Everett MJ, Jin YF, Ricci V, et al. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother* 1996;40:2380–2386.
10. Piddock LJ. Mechanisms of fluoroquinolone resistance: an update 1994–1998. *Drugs* 1999;58(suppl 2):11–18.
11. Heisig P. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 1996;40:879–885.

12. Giraud E, Leroy-Sétrin S, Flaujac G, et al. Characterization of high-level fluoroquinolone resistance in *Escherichia coli* O78:K80 isolated from turkeys. *J Antimicrob Chemother* 2001;47:341–343.
13. Conrad S, Oethinger M, Kaifel K, et al. *gyrA* mutations in high-level fluoroquinolone-resistant clinical isolates of *Escherichia coli*. *J Antimicrob Chemother* 1996;38:443–455.
14. Vila J, Ruiz J, Marco F, et al. Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. *Antimicrob Agents Chemother* 1994;38:2477–2479.
15. Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. *Curr Med Chem* 2009;16:1028–1046.
16. Robicsek A, Strahilevitz J, Jacoby GA, et al. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* 2006;12:83–88.
17. Yamane K, Wachino J, Suzuki S, et al. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrob Agents Chemother* 2007;51:3354–3360.
18. Piddock LJ. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* 2006;19:382–402.
19. Sulavik MC, Houseweart C, Cramer C, et al. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob Agents Chemother* 2001;45:1126–1136.
20. Thanassi DG, Cheng LW, Nikaido H. Active efflux of bile salts by *Escherichia coli*. *J Bacteriol* 1997;179:2512–2518.
21. Mazzariol A, Tokue Y, Kanegawa TM, et al. High-level fluoroquinolone-resistant clinical isolates of *Escherichia coli* overproduce multidrug efflux protein AcrA. *Antimicrob Agents Chemother* 2000;44:3441–3443.
22. Kriengkauykiat J, Porter E, Lomovskaya O, et al. Use of an efflux pump inhibitor to determine the prevalence of efflux pump-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2005;49:565–570.
23. Meunier D, Acar JF, Martel JL, et al. A seven-year survey of susceptibility to marbofloxacin of pathogenic strains isolated from pets. *Int J Antimicrob Agents* 2004;24:592–598.
24. Clinical and Laboratory Standard Institute. *Performance standards for antimicrobial disk and dilution susceptibility testing for bacteria isolated from animals; approved standard. 3rd ed.* CLSI Document M31–A3. Wayne, Pa: Clinical and Laboratory Standard Institute, 2008.
25. Shaheen BW, Wang C, Johnson CM, et al. Detection of fluoroquinolone resistance level in clinical canine and feline *Escherichia coli* pathogens using rapid real-time PCR assay. *Vet Microbiol* 2009;139:379–385.
26. Willmott CJ, Maxwell A. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrob Agents Chemother* 1993;37:126–127.
27. Lomovskaya O, Warren MS, Lee A, et al. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob Agents Chemother* 2001;45:105–116.
28. Drlica K. The mutant selection window and antimicrobial resistance. *J Antimicrob Chemother* 2003;52:11–17.
29. Randall LP, Cooles SW, Piddock LJ, et al. Mutant prevention concentrations of ciprofloxacin and enrofloxacin for *Salmonella enterica*. *J Antimicrob Chemother* 2004;54:688–691.
30. Oethinger M, Kern WV, Jellen-Ritter AS, et al. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. *Antimicrob Agents Chemother* 2000;44:10–13.
31. Lomovskaya O, Lee A, Hoshino K, et al. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1999;43:1340–1346.
32. Baucheron S, Imberechts H, Chaslus-Dancla E, et al. The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium phage type DT204. *Microb Drug Resist* 2002;8:281–289.
33. Luo N, Sahin O, Lin J, et al. In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. *Antimicrob Agents Chemother* 2003;47:390–394.
34. Nishino K, Yamaguchi A. Analysis of the complete library of putative drug transporter genes in *Escherichia coli*. *J Bacteriol* 2001;183:5803–5812.
35. Rosenberg EY, Ma D, Nikaido H. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. *J Bacteriol* 2000;182:1754–1756.
36. Lomovskaya O, Watkins W. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. *J Mol Microbiol Biotechnol* 2001;3:225–236.

Appendix 1

Antimicrobial drugs used in a study of the contribution of *gyrA* mutation and efflux pumps to fluoroquinolone resistance and MDR in pathogenic *Escherichia coli* isolates in dogs and cats.

Drug class	Antimicrobial drug	MIC_{BP} (S,R [mg/L])	MIC range (mg/L)
β-lactams	Ampicillin	≤ 8, ≥ 32	0.5–256
Penicillins	Amoxicillin-clavulanic acid	≤ 8/4, ≥ 32/16	0.25–512
	Ticarcillin-clavulanic acid	≤ 16/2, ≥ 128/2	2–2,048
Carbapenem	Meropenem	≤ 4, ≥ 16	0.25–512
Cephalosporins	Cephalothin	≤ 8, ≥ 18	1–2,048
	Cefoxitin	≤ 8, ≥ 32	0.5–2,048
	Cefpodoxime	≤ 2, ≥ 8	0.12–128
	Cefotaxime	≤ 8, ≥ 64	1–1,024
	Ceftazidime	≤ 8, ≥ 32	0.5–512
Fluoroquinolones	Enrofloxacin	≤ 0.5, ≥ 4	0.06–128
Tetracycline	Doxycycline	≤ 4, ≥ 16	0.25–512
Phenicols	Chloramphenicol	≤ 8, ≥ 32	0.5–1,024
Macrolides	Azithromycin	≤ 0.5, ≥ 8	0.12–256
Aminoglycosides	Gentamicin	≤ 4, ≥ 8	0.12–256
Potentiated sulfonamides	Trimethoprim-sulfamethoxazole	—	0.06–12

— = Not applicable.
MIC_{BP} = MIC break point. S = Susceptible. R = Resistant.

Appendix 2 appears on the next page

Appendix 2

Primers and probes used in a FRET-PCR assay and melting curve analysis in a study of the contribution of *gyrA* mutation and efflux pumps to fluoroquinolone resistance and MDR in canine and feline pathogenic *E coli* isolates.

Primer or probe	Oligonucleotide sequence (5′-3′)	Position*	T*m* (°C)
Upstream primer	CCATGAACGTACTAGGCAATGACTG	152–177	57
Downstream primer	TTTTCCGTGCCGTCATAGTTATCAA	256–427	58
Fluorescein	GTTGGTGACGTAATCGGTAAATACCATCCCC-(6-FAM)	208–238	66
Bodipy 630/650	(Bodipy-630/650)-TGGTGACTCGGCGGTTTATGACACGA-(Phosphate)	240–265	66.8

*Positions of the oligonucleotides correspond to those in GenBank accession No. X06373.
6-FAM = 6-Carboxyfluorescein. T*m* = Theoretical melting temperature.