THE ROLE OF *GYRA*, EFFLUX PUMP AND INTEGRONS IN MEDIATING THE EMERGENCE OF MULTI-DRUG RESISTANCE AMONG CANINE AND FELINE PATHOGENIC CLINICAL *ESCHERICHIA COLI*

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Bashar Wajeeh Shaheen

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VITA

Bashar W. Shaheen, son of Wajeeh Raghib Shaheen and Aziza Abdulfatah Naser, was born August 29, 1975, in Kuwait, State of Kuwait. He graduated from comprehensive high school in 1993. He completed his degree of Bachelor of Veterinary Medicine and Surgery (BVMS) equivalent to DVM from Mosul University, Iraq in September, 1998. After working as a veterinarian in Greater Amman Municipality (food safety and inspector in charge) for three years, he immigrated to the United States and entered Graduate School in the Department of Poultry Science at Auburn University, Alabama in January 2004 and graduated with a Master of Science degree in May, 2006. In 2006, he began his Doctoral work in Biomedical Science at Auburn University College of Veterinary Medicine.

DISSERTATION ABSTRACT

THE ROLE OF *GYRA*, EFFLUX PUMP AND INTEGRONS IN MEDIATING THE EMERGENCE OF MULTI-DRUG RESISTANCE AMONG CANINE AND FELINE PATHOGENIC CLINICAL *ESCHERICHIA COLI*

Bashar Wajeeh Shaheen

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A series of experiments was performed to elucidate the emergence of fluoroquinolone (FQ)/multi-drug resistance (MDR) and the contribution of selected mechanisms of resistance to MDR (i.e. mutations in *gyrA*, efflux pump, and the integrons) in canine and feline pathogenic *Escherichia coli*. *E. coli* isolates (n= 377) were collected from dogs or cats with spontaneous infection between May and September 2005 for phenotypic (i.e. E-test®) and genotypic (i.e PFGE) characterization. Of isolates expressing resistance to any drug, two isolates expressed single drug resistance (SDR) only to enrofloxacin. The remaining 109 isolates expressing resistance to enrofloxacin also expressed MDR, with resistance to all 7 drugs (Z phenotype) representing the largest proportion (18.3%; 20/109). The drugs most commonly involved in MDR phenotypes (n=109) were amoxicillin (96.3 %; 105/109), amoxicillin-clavulanic acid (85%; 93/109)

and enrofloxcin (61.5%; 67/109). Genotypically, isolates were extensively diverse, regardless of resistance phenotype, and phenotypes and genotypes were not related.

For the first time, a FRET PCR was developed to identify enrofloxacin-resistance in clinical *E. coli* isolates that carry mutations in codon 83 and 87 of *gyrA*. The assay identified as few as four genome copies per reaction from culture and 19 genome copies in urine with a very short time (1-2 hrs). For the 70 isolates tested, the sensitivity of the test was 87.5% (95% CI = 75% to 95.3%) (n=42/48) whereas specificity was 100% (95% CI = 87.3% to 100%) (n=22/22). MICs for *E. coli* isolates (n= 536) were determined for enrofloxacin and five other drug classes using broth micro-dilution. FQ resistance was significantly associated with the MDR phenotype compared to non FQ-R isolates. The results suggest that the double mutation in *gyrA* confers high level of resistance to the majority of FQ-R isolates. Furthermore, the impact of an efflux pump was studied. Blocking the action of the AcrAB efflux pump (i) decrease the intrinsic level of the MICs to FQ in susceptible isolates, (ii) decrease the MICs below the susceptible break point for FQ-R isolates even with single mutation in *gyrA*, (iii) decrease the magnitude of resistance in highly resistance isolates in the presence of double mutations in *gyrA* (IV) decrease the MICs below the susceptible break point for some of the highly FQ-R isolates without *gyrA* mutations, and (V) decrease the resistance to structurally unrelated drugs, thus reducing the incidence of MDR. Integrons of Class I and 2 were identified in 27% of the isolates; thus only 2.4% of the isolates carried Class 2 integrons. All integron positive isolates were MDR, compared to 56.6% of integron negative isolates. Resistance resolved in the 3 MDR integron positive isolates subjected to plasmid curing (using 10 % SDS). This is the first report of class 2 integrons in *E. coli* from companion animals in US.

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I would like to thank my parents, my father Wajeeh Shaheen and my mother Aziza Naser, who taught me the value of hard work by their own experience. I would like to share this moment of happiness with my father and mother-in law "Hafez and Khawla", who rendered me enormous support during the whole study. Finally, and most importantly, I would like to thank my wife Suhair. Her support, encouragement, quiet patience and unwavering love were undeniably the bedrock upon which the past eight years of my life have been built. Her tolerance of my occasional absence is a testament in itself of her unyielding devotion and love. Without her love, support and understanding I would never have completed my present work. Particularly, I owe to my son Kareem and my daughter Sarah for their silent prayer for my work to be successfully completed.

Style manual or journal used

Journal of Veterinary Microbiology

Computer software used

Microsoft® Windows® XP Professional

Statistical software MINITAB® 15 package

Vector NTI™ Advance 10

SWIN® Epidemiology Module

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CHAPTER I

LITERATURE REVIEW: *ESCHERICHIA COLI* AND FLUOROQUINOLE RESISTANCE IN COMPANION ANIMALS

Fluoroquinolone resistance among canine and feline *E. coli*

Escherichia coli is an important pathogen associated with urogenital and other infections in dogs and cats. An increase in the rate of resistance to fluoroquinolones has been reported among *E. coli* isolates from dogs and cats (Cohn *et al.,* 2003; Cooke *et al.,* 2002; Guardabassi *et al.,* 2004). Cooke *et al.* (2002) reported an increase in enrofloxacin-resistant *Escherichia coli* associated with urinary tract infections in dogs at a veterinary medical teaching hospital and from other veterinary hospitals in California. Those enrofloxacin-resistant *Escherichia coli* isolates were also resistant to other antimicrobials such as ampicillin, trimethoprim-sulphamethoxazole, and cephalexin. The trend of increased resistance to fluoroquinolone among uropathogenic *E. coli* isolates from dogs between 1992 and 2001 in Missouri was determined for 1,478 bacterial isolates including *Escherichia coli* (n=547 isolates). The overall resistance of *E. coli* (n=547) to fluoroquinolones increased significantly over time ($P < 0.0001$). For each drug, the increased resistance of *E. coli* to ciprofloxacin was significant ($P = 0.0001$), but resistance to enrofloxacin failed to reach statistical significance ($P = 0.022$ at significant P value of 0.01). Boothe *et al.* (2006) also studied the pharmacodynamic and

pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. Percent resistance for all Gram-negative and Gram-positive isolates did not differ among drugs or organisms; however *E. coli* resistance to fluoroquinolones approximated 40%. Resistance contributed to a bimodal distribution of MIC, with the MIC 50 at ≤ 0.25 mcg/ml, but the MIC 90 ≥ 32 μg/ml. Similarly, studies from human medicine indicated the association of fluoroquinolone with multidrug-resistant phenotypes commonly with the clinical cases of uropathogenic *E. coli* (Karlowsky *et al.,* 2002, 2003, 2006).

previous work of Boothe et al indicated a high incidence of MDR in *E. coli* uropathogens isolated from canine patients admitted to Auburn University's Veterinary Teaching Hospital (n=175). Only 50% of uropathogens were *E. coli.* Overall *E. coli* resistance to all 17 antimicrobials was 31% and approximated 40% to 50% toward "first choice" empirical antimicrobials (amoxicillin, amoxicillin-clavulanic acid, cephalexin and sulfonamide-trimethoprim) and toward enrofloxacin (Boothe *et al.,* 2005). Furthermore our pilot data indicated that fluoroquinolone-resistant isolates (i.e. those resistance to enrofloxacin) were more likely to be multi-drug resistant (MDR). Only 2 out 85 (2%) isolates expressed single drug resistant (SDR) phenotype, while (61.5%; n=67/109) of MDR phenotypes were associated with enrofloxacin resistance (Shaheen *et al.,* 2008a, 2008b).

An important risk factor associated with the emergence of fluoroquinolones resistance is the approval and the possibility of increased use of broad-spectrum antimicrobials (i.e. fluoroquinolones) at veterinary hospitals as previously described (Sanchez *et al.,* 2002). Furthermore, the enrofloxacin resistant isolates were also

associated with resistance to other antimicrobial agents. This association was also observed at a human hospital setting (Richard *et al.,* 1994) especially with patients who had a long term treatment with ciprofloxacin (Muder *et al.,* 1991). As consequence, the emergence of resistance to *E. coli* and treatment failure has been observed during treatment of *E. coli* infections with quinolines (Troung *et al.,* 1995). Emergent resistance in *E. coli* or other bacterial organisms in animals receiving fluoroquinolones might present a public health risk, as has been demonstrated thorough transferring resistant isolates from food producing animals previously treated with FQs (Webber and Piddock, 2001). For dogs and cats, the risk may be increased through the close physical contact between household pets and humans (3). Indeed, this is supported by the fact that resistance mechanisms (i.e. resistance genes) in these isolates are the same as those in the resistant strains found in humans (Guardabassi *et al.,* 2004, Webber and Piddock, 2001).

The antimicrobial action of fluoroquinolones

Quinolones interact with two type II topoisomerases (bacteria DNA gyrase) and topoisomerase IV. Type II topoisomerases act by breaking both strands of duplex DNA, passing another DNA strand through the break, and resealing the gap. DNA gyrase is a tetrameric enzyme composed of two gyrA subunits and two gyrB subunits. It maintains the DNA structure through catalyzing the negative supercoiling of DNA (Reece and Maxwell, 1991). Negative supercoils are important for initiation of DNA replication and facilitate binding of initiation proteins. During transcription, the replication fork and RNA polymerase tend to introduce positive supercoils which must be removed to avoid stalling of the replication fork (Hooper, 1999). Topoisomerase IV is also a tetrameric

enzyme consisting of two ParC and two ParE subunits. Its function is important during the DNA replication by segregation of replicated daughter chromosomes (Drlica and Zhao, 1997). Whereas both DNA gyrase and topoisomerase IV can remove positive supercoils, only DNA gyrase can convert them directly to negative supercoils.

The mechanisms of resistance to fluoroquinolones

The common mechanism of action of fluoroquinolone is inhibition of topoisomerase. This is demonstrated by point mutations that result in amino acid substitutions within the topoisomerase genes, accompanied with decreased expression of outer membrane porins and overexpression of multidrug efflux pumps. In addition, the discovery of plasmid-mediated quinolone resistance suggests that they which could play a role in horizontal transfer of resistance genes between strains. In addition to that is the discovery of plasmid-mediated quinolone resistance which could play a role in horizontal transfer of resistance genes between strains. Table 1 summarizes the common mechanisms of resistance to quinolone and fluoroquinolone. It is should be noted that mutations in topoisomerase genes cause irreversible changes that will be inherited by all daughter cells of the resistant isolate. This can be a cause of the therapeutic failure due to the emergence of large populations of resistant bacteria. In contrast, plasmid mediated resistance is reversible by removal of the antimicrobial agent, resulting in a loss of positive selective pressure for those bacteria carrying the plasmid.

Table 1. Resistance to fluoroquinolones by different mechanisms. [a]C, chromosomal DNA; P, plasmid; I, integron.

Resistance mechanism	Resistance gene(s)	Gene product(s)	Bacteria involved	Location of Resistance gene[a]
Mutational modification of the target site	Mutation in gyrA resulting in changes of Ser-83 (to Tyr, Phe, or Ala) and Asp-87 (to Gly, Asn, or Tyr) were detected most commonly. Stepwise mutations in *gyrA* and *parC* can result in an incremental increase resistance to fluoroquinolones.	Mutation in the genes for DNA gyrase and topoisomerase IV	Various gram-positive and –negative bacteria	**C**
Mutational modification of regulatory genes that control the efflux pump systems	The level of production of the AcrAB-TolC efflux system in E. coli is under the control of several regulatory genes (*marRAB*, *soxR*, or *acrR* genes). Mutation in these regulatory genes leads to overproduction of the AcrAB-TolC efflux pump and the expression of MDR phenotypes.	Mutation in the *marRAB*, *soxR*, or *acrR* genes	*E. coli*	**C**
Efflux via multidrug transporters	*mexB-mexA-oprM, acrA-acrB-TolC*	Multidrug efflux in combination with specific outer membrane proteins	*Pseudomonas, E. coli, Salmonella*	**C**
Decreased drug uptake	OmpF is an important porin for the entery of quinolones and fluoroquinolones into the bacterial cell.	MAR-mediated down regulation of OmpF porin	Gram-nagative bacteria	**C**

		production. Mutation in different gene loci (*cfxB, norB, nfxB, norc, or nalB*) are also associated with decreased permeability.		
DNA gyrase protection	Low level quinolone resistance via protection of DNA gyrase from inhibition by quinolones is mediated by the *gyr* gene.	*Qnr* gene	*K. pneumoniae*, and *E. coli*	**P, I**

Mutations in the topoisomerase genes

The mutations in DNA gyrase and topoisomerase IV are the two important mechanisms that confer resistance to the quinolone. Many studies have reported that topoisomerase IV in gram-negative bacteria is the secondary target for quinolones (Khodursky *et al.,* 1995; Kumagai *et al.,* 1996; Heisig, 1996), and the primary target for gram positive bacteria (Ferrero *et al.,* 1994). Fluoroquinolones inhibit the DNA synthesis through stabilizing the breaks in the DNA made by the DNA gyrase or topoisomerase IV. Furthermore, amino acid changes in the quinolone resistance-determining region (QRDR) of GyrA alter the structure of the site of quinolone binding near the interface of the

enzyme and DNA and that resistance is then caused by reduced drug affinity for the modified enzyme-DNA complex.

Mutation in DNA gyrase

Most mutations have been described previously are located in a region called the quinolone resistance-determining region (QRDR). The region (Fig. 1) in *E. coli* is located between amino acids Ala67- and Gln106 in *gyrA* (Yoshida *et al.,* 1990) and at amino acid Asp426 and Lys447 in *gyrB* (Yoshida *et al.,* 1991). Mutations occur mostly at codons 83 and 87, which changes the binding of quinolones to the active site and confers resistance to quinolones (Willmott and Maxwell, 1993).

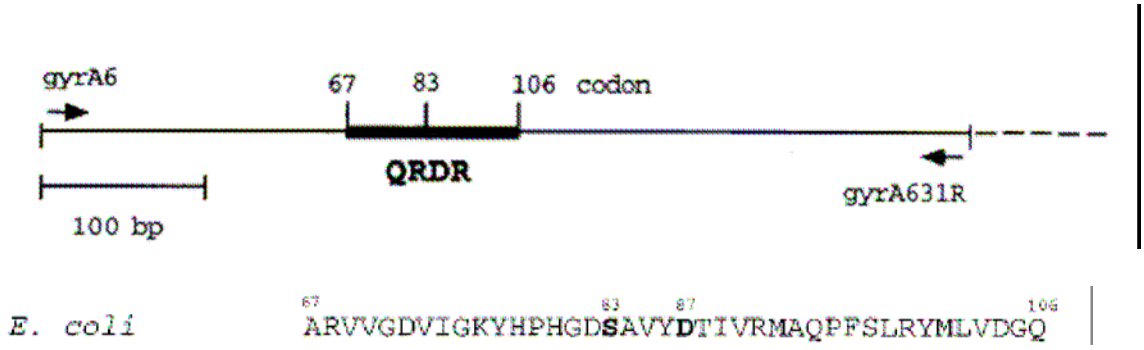

Figure 1. Schematic representation of the *gyrA* region amplified by primers gyrA6 and gyrA631R (arrows), including the 120-bp QRDR (heavy line) encoding amino acids 67 to 106 of the *E. coli* GyrA protein (Swanberg and Wang, 1987)

DNA gyrase mutations in *E. coli*

The level of resistance to fluoroquinolone is condon specific and can be affected by the number of the mutations involved. For example, a single mutation in *gyrA* in *E. coli* conferred high-level resistance to nalidixic acid (Markham and Neyfakh, 1996; Luo

et al., 2006). However, further mutations in *gyrA* and/or the topoisomerase IV genes are essential to confer high-level resistance to fluoroquinolone (Everett *et al.,* 1996; Ozeki *et al.,* 1997; Weigel *et al.,* 1998). Clinically, including the veterinary strains of *E. coli,* most mutations in *gyrA* occur at codon 83. In *E. coli* the mutation at codon 83 involves most commonly the substitution of serine residue for a leucine which confers high-level resistance to nalidixic acid and fluoroquinolones (Ozeki *et al.,* 1997; Weigel *et al.,* 1998; White *et al.,* 2000). Further mutation at codon Asp87 generates slightly higher resistance to fluoroquinolones (Lehn *et al.,* 1996; Sáenz *et al.,* 2003). In contrast, the single mutation at Ser83 has its greater impact on the isolate to become more resistant to fluoroquinolones than those with a single mutation at Asp87 or other regions (Lehn *et al.,* 1996). Many studies reported that single mutations in Gly81Asp, Asp82Gly, Asp87Asn, Asp87Gly and Asp87Tyr result in low-level resistance to fluoroquinolones (McDonald *et al.,* 2001). In general the mutations at codons Ser83 and Asp87 confer higher levels of quinolone resistance than mutations in other codons (Yoshida *et al.,* 1990).

Mutation in Topoisomerase IV genes

Many studies indicated that topoisomerase IV plays a secondary role as a target for quinolones in Gram-negative bacteria (Hoshino *et al.,* 1994; Kato *et al.,* 1992; Khodursky *et al.,* 1995; Peng and Marians, 1993). Other studies found that mutations in *parC* and *parE* of *E. coli* always occurred in conjunction with mutations in *gyrA* (Everett *et al.,* 1996; Komp Lindgren and Karlsson, 2003; Fendukly *et al.,* 2003; Sáenz *et al.,* 2003). These findings suggested that the DNA gyrase mutations should proceed the topoisomerase IV mutations to confer high-level fluoroquinolone resistance in *E. coli*

(Everett *et al.,* 1996; Heisig, 1996). Another in vitro study indicated that single mutation within *parC* did not confer the strain to be resistant to the quinolone and that expression to level resistance to fluoroquinolone was achieved by a combination of the *gyrA* double mutation and one *parC* mutation (Bagel *et al.,* 1999). Another in vitro study revealed that mutation in *parC* was the second step confering high-level resistance to fluoroquinolone in *Escherichia coli* (Bachoual *et al.,* 1998). Therefore mutations topoisomerases IV genes impact the level of resistance to fluoroquinolones.

The advantages of real-time PCR

For our clinical purposes, utilized the real time PCR has major advantages for detection of mutations in codon 83 and 87 of *gyrA* in *E. coli*. The LightCycler (Roche, Inc. Indianapolis, IN) System has been designed to reduce the time required to achieve results from PCR reactions and to monitor the amplification of the target sequence simultaneously, in real-time. The system has an advantage to maximize the amount of information that can be obtained from a single reaction, making the system a powerful tool for quantitative PCR and mutation analysis. A major benefit of using this system is the short cycling time; so that a complete PCR run of 20 -30 cycles can be performed in 20 – 30 minutes. Furthermore, because no gel electrophoresis is required, contamination was minimized as result of maintaing tubes sealed at the end of the amplification.

Fluorescence Resonance Energy Transfer (FRET)

The hybridization probe format was utilized for maximum sequence-specificity for detection of PCR product. Two designed, sequence-specific oligonucleotide probes, labeled with different dyes, were used. The sequence of the two probes can hybridize to

the target sequences on the amplified DNA fragment in a head-to-tail arrangement. The donor dye (Fluoroescein) is excited by the blue light source and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the energy emitted excites the acceptor dye attached to the second Hybridization Probe, which then emits fluorescent light at a different wavelength. This energy transfer, referred to as fluorescence resonance energy transfer (FRET) is highly dependent on the close proximity spacing between the two dye molecules (1-5 nucleotides). The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process.

Typing of nucleotide sequence polymorphisms and analyzing mutations

The LightCycler System can achieve precise discrimination of DNA sequence by monitoring the temperature-dependent hybridization of sequence-specific hybridization probe to single stranded DNA while performing the unique melting curve analysis. The melting temperature (T_m) is dependent on the probe length and GC content, and on the degree of homology between the two DNA strands. Hybridization probes bound perfectly to the matching target DNA which required a higher T_m to separate in comparison with those bound to DNA containing destabilizing mismatches (i.e. the oligonucleotide and its perfect complement are in duplex). This is a powerful tool in which no post-PCR processing is required, and thus minimizes the possibility of contamination as the amplification and genotyping will be performed in the same sealed capillary.

In the mutation analysis experiment, one hybridization probe was labeled with an LC Red flourophore which hybridized to a part of the target sequence that was not

mutated and which will function as an anchor probe. The other hybridization probe, will be labeled with a Fluorescein fluorophore and, in the mutated isolates, spanned the (shorter [mutation probe]) mutation site (Fig 2). After hybridization, the two DNA probes come into close proximity, resulting in FRET between the two fluorophores. During the melting curve analysis, as increasing temperature caused the shorter mutation probe to dissociate first, and the two fluorescent dyes were no longer in close proximity, causing fluorescence to decrease. If the mutation was present, the mismatch of mutation probe with the target (anchor) will destabilize the hybrid, causing T_m to be lower. In contrast, mismatches did not occur with wild type phenotype, and the heteroduplex will thus have a higher T_m (Fig 4). An example is using the FRET probe assay to differentiate *Y. pestis* KIM 5 *gyrA* mutants (Cp^r) from wild-type (Cp^s) organisms (Lindler *et al.,* 2001). As shown in Fig. 8, the melting peak temperature was dependent on the homology between probe 1 and the amplified PCR product. All of the mutant PCR products formed a less-stable hybrid with the designed probe 1 than did the wild-type *gyrA* sequence. The largest difference in melting peak was seen with the cytosine-to-adenosine mutation (M4 in Fig. 3). The two mutations, which were the most distal to the 3' end of probe 1 (M1 and M2 in Fig. 4), had an intermediate T_m. The mutant *gyrA*-probe1 T_m was easily differentiated from those generated with wild-type templates. The minimum T_m decrease compared to that for the wild-type sequence was with mutant M3, for which the T_m was around 3°C (Fig. 3). The maximum T_m shift compared to *Y. pestis* KIM 5 was around 10°C for the M4 mutant.

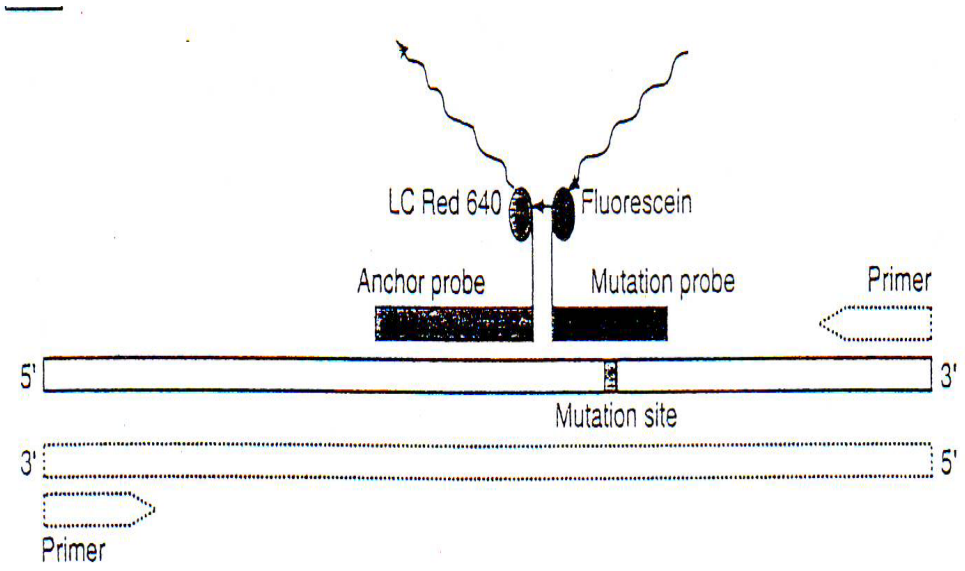

Figure 2. Fluorescence resonance energy transfer (FRET). The mutation probe (Fluorescein) spans the mutation site while the anchor probe hybridizes to a part of the target sequence that is not mutated. FRET occurs when the two probes hybridizing to the target sequence come in close proximity (1-5 nucleotides apart). The energy emitted excites LC640, which then emits red fluorescent light.

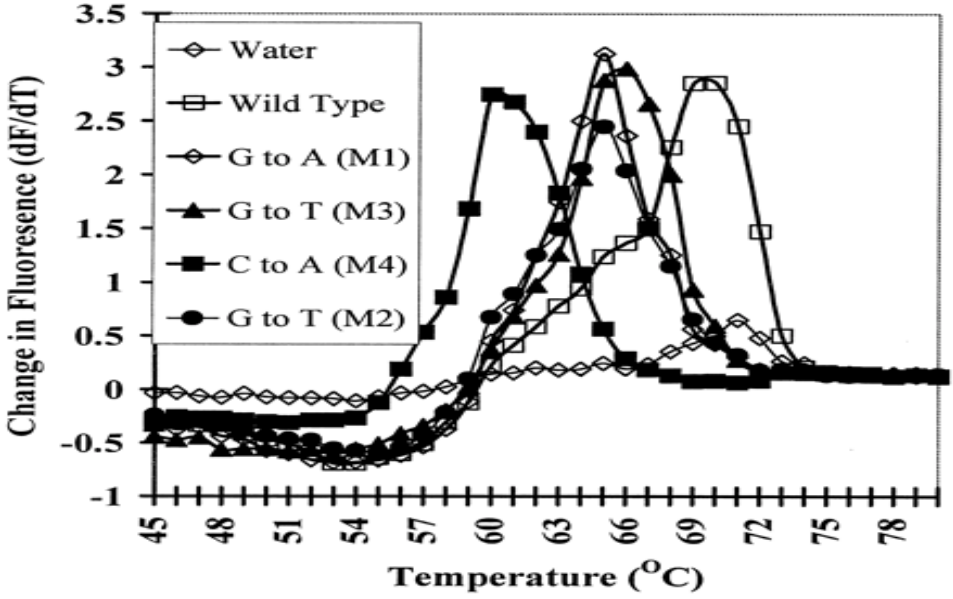

Figure 3. Using FRET to differentiate the mutant and the wild-type of *Y. pestis*. Wild-type and mutant templates were used in the PCR amplification followed by melting peak analysis. (Lindler *et al.,* 2001).

Efflux pump system

Five different families of efflux pump proteins (Fig. 4) have been identified in gram positive and negative bacteria and these include the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), and the staphylococcal multi-resistance (SMR), multidrug and toxic compound extrusion (MATE) families, and ABC (ATP binding cassette). The efflux pump systems of the RND family are organized as tripartite efflux pumps. The pump is very important in *Escherichia coli* and other gram-negative bacteria (e.g., *E. coli acrB*/AcrB, *P. aeruginosa mexB*/MexB, *Campylobacter jejuni cmeB*/CmeB, and *Neisseria gonorrhoeae mtrD*/MtrD). The efflux pump has three components: a transporter (efflux) protein in the inner membrane (e.g., AcrB), a periplasmic accessory protein (e.g., AcrA), and an outer membrane protein channel (e.g., TolC) (Koronakis *et al.,* 2004). AcrB captures its substrates within either the phospholipid bilayer of the inner membrane of the cytoplasm (Aires and Nikaido, 2005) and effluxes them into the external medium via TolC. The AcrB and TolC are connected via the periplasmic protein AcrA. AcrA protein provides collaboration between AcrB and TolC. The RND pumps are proton antiporters, using the proton gradient across the membrane to power efflux, exchanging one H^+ ion for one drug molecule (Paulsen, 2003).

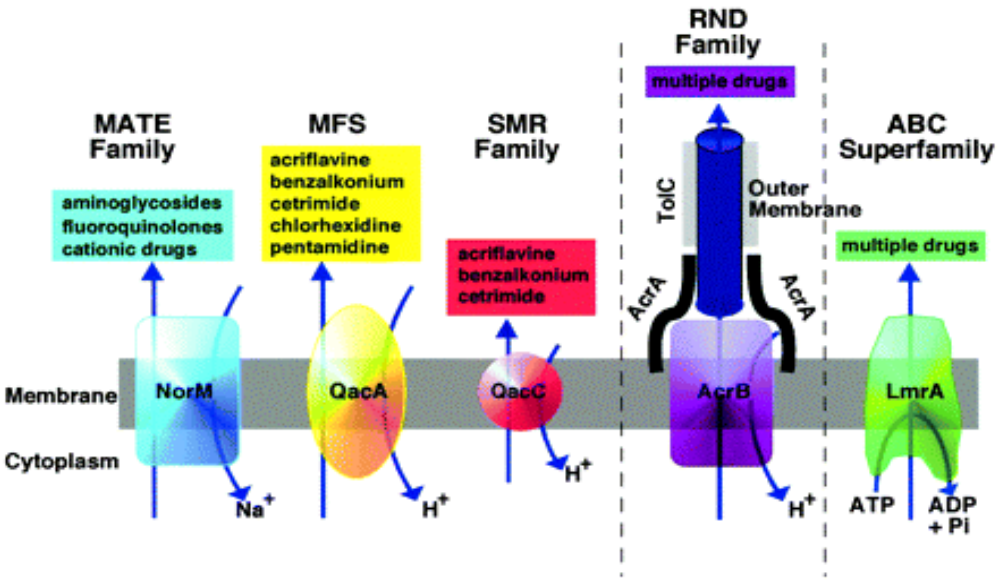

Figure 4. Diagrammatic comparison of the five families of efflux pumps (Piddock, 2006).

The AcrAB-TolC system as an example of RND system in *E. coli* has wide range of substrates includes chloramphenicol, lipophilic ß-lactams, fluoroquinolones, tetracycline, rifampin, novobiocin, fusidic acid, nalidixic acid, ethidium bromide, acriflavine, bile salts, short-chain fatty acids, SDS, Triton X-100, and triclosan (Folster and Shefer, 2005; Nikaido, 2003; Olliver *et al.,* 2004; Tegos *et al.,* 2002; Wolter *et al.,* 2004). Overexpression of AcrAB-TolC system alone or mutations in the topoisomerase gene is unlikely, alone, to give rise to clinical levels of resistance to certain drugs such as ciprofloxacin, chloramphenicol, tetracycline, and cotrimoxazole (Piddock, 2006; Tegos *et al.,* 2002). This is possibly because the resistance was caused by systems other than AcrAB (e.g. mutations in gyrase or topoisomerase IV) (Tegos *et al.,* 2002). However, many studies suggest that the combination of a mutation in the drug binding site gene with over expression of an efflux pump give rise to high level of resistance to

fluoroquinolones (Mazzariol *et al.,* 2000, 2002; Oethinger *et al.,* 1998). The over expression of efflux pumps may confer the bacterium to become resistant to antimicrobials. In one study, reserpine (Inhibitors of MDR Efflux Pumps) was shown to suppress the in vitro emergence of norfloxacin-resistant *S. aureus* (Markham and Neyfakh, 1996) and ciprofloxacin-resistant *S. pneumoniae* (Markham, 1999). The efflux pump inhibitor MC-207-110 also suppressed the emergence of levofloxacin-resistant *P. aeruginosa* (Lomovskaya *et al.,* 2001).

One study indicated that deletion of *acrAB* in *E. coli* with two mutations in *gyrA* resulted in increasing the susceptibility to fluoroquinolones. Inactivation of *acrB* in MDR *S. enterica* serovar Typhimurium DT204 (with a mutation[s] in topoisomerase genes) resulted in very low MICs (even below the break points). Similar results observed after deletion of *cmeB* in *C. jejuni* containing a substitution in GyrA (Luo *et al.,* 2003). Furthermore, in vitro mutant *S. enterica* serovar Typhimurium lack *tolC* were unable to become resistant to ciprofloxacin (Ricci *et al.,* 2006).

Integrons

Integrons "are natural genetic engineering systems that scavenge and incorporate circularized open reading frames, called gene cassettes, and convert them to functional genes" (by ensuring their correct expression) (Rowe-Magnus and Mazel, 2002). In the integrons, the incorporation of multiple genes into gene cassette creates the problem of multidrug resistance. The integrons can contain up to 8 resistance gene cassettes and have been found in multiple- resistant clinical isolates. More than 80 different antibiotic resistance genes, covering most classes of antimicrobials presently in use, are identified

in class 1 integrons. These include genes conferring resistance to know beta-lactams, all aminoglycosides, chloromphenicol, trimethoprim, streptothricin, fosfomycin, rafampin, erythromycin, lincomycin, and antiseptic of quaternary ammonium compound family.

The integron platform consists of an integrase gene and a primary recombination site, called the attI site proximal to the integrase gene (Mazel, 2006). The integrase catalyzes recombination between its cognate attI site and a secondary target called an attC site (or 59 base elements). The attC site is normally found associated with a single open reading frame (ORF).The combination structure of the attC-ORF is termed a gene cassette. Insertion of the gene cassette at the *attI* site, which is located downstream of the common promoter internal to the *intI* gene, obliges expression of the down stream gene cassettes (Fig 5) (Fluit and Schmitz, 2004).

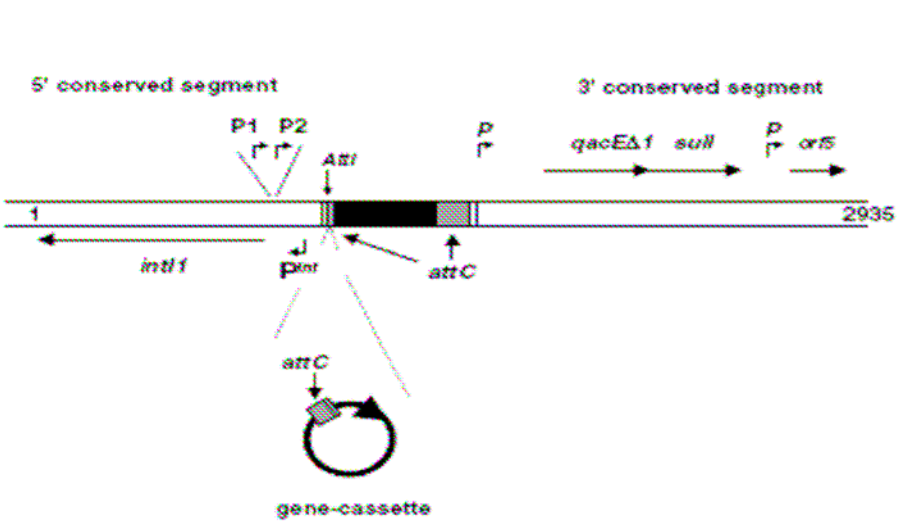

Figure 5. General structure of class 1 integron. P1, promoter for trancription of gene cassettes; P2, second promoter that is usually inactive; int, integrase gene; attI1, integration site; qacE, partially deleted gene that encodes quaternary ammonium compound resistance; sulI, sulphonamide resistance; orf5, unknown function; P, promoters of the qacED and sulI genes; attC, sequence on the gene cassette recognised by the integrase (Fluit and Schmitz, 2004).

Five classes of multi-resistant integrons (MRIs) share the same pool of resistance cassettes (Rowe-Magnus and Mazel, 2002). For example, the dfrA1 cassette has been identified in the Class 1, 2, 4 and 5 integrons of various species. Each of the five classes of mobile integrons was defined based on the homology of their integrase genes, which share between 45 - 58% amino acid identities. The integron platforms are found in a variety of genetic contexts. All 5 classes linked to mobile genetic elements; all of which serve as vehicle for inter and intra microbial species transmission of genetic material. The class 1 integron platform is the most common among multi-drug resistant bacterial populations and is found associated with and can be embedded in the Tn21 transposon family. Class 1 integrons have been reported in many Gram negative genera including Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Campylobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Pseudomonas, Salmonella, Serratia, Shigella and Vibrio. It has also been identified in other bacteria such as Corynebacterium glutamicum and Mycobacterium fortuitum, and Enterococcus faecalis (Mazel, 2006). The other 3 classes of integrons are associated with different Tns. Class 2 integrons are embedded in the Tn7 family of transposons and consist of an integrase gene followed by gene cassettes. Class 2 integrons is not as common as class 1 integrons and found in Acinetobacter, Shigella and Salmonella. One observation is that the *intI2* gene of Tn7 contains a stop codon and Tn7 cannot therefore produce an active integrase. Thus may explain the high frequent detection of class 1 integrons rather than class 2 among clinical isolates. Class 3 integrons have been described in Pseudomonas aeruginosa, Serratia marcescens, Alcaligenes xylosoxidans, Pseudomonas putida and Klebsiella pneumoniae

isolates from Japan. Class 4 is now named Vibrio cholerae SI and is a component of the SXT element (a conjugative self-transmissible integrating element) in this species (Mazel, 2006). The other (class 5) is located in compound transposon carried on a plasmid of *Vibrio salmonicida* (Mazel, 2006). It has been found that class 4 and 5 integrons harbor only a single antibiotic resistance cassette followed by several other cassettes with no antibiotic resistance function.

Research Objectives

The main aim of the present dissertation was to investigate the mechanisms by which muti-drug resistance (MDR) phenotypes emerge in association with enrofloxacin resistance in canine and feline clinical *E. coli* pathogens associated with spontaneous infections. The study was approached in four phases.

Chapter II is a preliminary study to generate baseline data on the prevalence and molecular basis of antimicrobial resistance in the canine and feline clinical *Escherichia coli* isolates. Isolates were collected from dogs or cats with spontaneous infection between May and September 2005. Isolates were phenotypically characterized based on the susceptibility to 7 antimicrobial agents by E-test® according to CLSI guidelines and interpretive standards. In addition, isolates from each phenotype were randomly selected for genotypical characterization using pulsed-field gel electrophoresis (PFGE).

Chapter III is to develop a real time- Fluorescence Resonance Energy Transfer (FRET)-PCR assay that is specifically designed to detect FQ resistant levels associated with the mutations in codon 83 and 87 of *gyrA* in ENR-resistant *E. coli* clinical isolates.

A muli-sequence alignment was initially performed on clinical *E. coli* isolates from which probes and primers were designed. Only one probe was designed that hybridizes perfectly *gyrA* sequence in wild type of clinical *E. coli* isolates. Melting curve analysis (MCA) was used to differentiate between the wild and the mutant-type template of *gyrA*. This was based on different melting peaks that emerge as a result of the mismatches between the probe and target sequences. Since this method is sensitive, rapid and specific, it can be used clinically in the future to detect these mutations and potential ENR-MDR phenptypes and can be used as an alternative for other conventional PCR techniques.

Chapter IV is to confirm and characterize the association of enrofloxacin (ENR) resistance with MDR in canine and feline pathogens. The resistance was described in terms of incidence (susceptible, intermediate and resistant) and phenotypes. The relationship between ENR resistance and various phenotypes was investigated. Pathogens collected from infected dogs and cats throughout the US were obtained from IDEXX (commercial veterinary microbiologic laboratory). For each isolate, the minimal inhibitory concentrations (MIC) were determined for 14 drugs, representing 6 different antimicrobial classes. Drugs were selected based on their historical use in veterinary medicine and their different mechanisms of action and thus resistance. Methods were based on micro-dilution susceptibility testing (TREK® Diagnostic Systems, Cleveland, OH); however, in contrast to standard methods, drug concentrations covered a range of over 500 fold differences, including concentrations well below and well above the breakpoint MIC as set by CLSI guidlines. The association between ENR resistant isolates

and the emergence of MDR resistant phenotypes was investigated using statistical methods of odds ratio and CI. Comparisons were made in proportion of co-resistance to other drug classes for isolates which do exhibit enrofloxacin resistance and the isolates that do exhibit resistance to all drugs but enrofloxacin. Furthermore, we investigated the contribution of resistance nodulation division (RND) efflux pump (AcrAB-TolC system in *E. coli*) to the emergence of ENR-MDR phenotypes. The role of the pump was confirmed and characterized by repeating susceptibility testing of all isolates, with the addition of the efflux pump inhibitor (EPI; Phe-Arg-ß-naphthylamide) to the culture media (100 μg/ml). Overexpression phenotype (EPO) was defined as a four fold or more decrease in the MIC for ENR when the inhibitor was present compared to its absence. The outcome measurements were included the statistical comparison of MIC statistics for each drug in the presence and absence of the EPI, and the magnitudes (how many fold decrease in MIC) of MIC decrease in the presence of EPI.

Chapter VI is to explore the association of mobile genetic elements class 1 and 2 integrons in the emergence of MDR phenotypes and among different phenotypes. This part of the dissertation is to answer the question about role of mobile genetic elements into the dissemination of antimicrobial resistance genes. Integrons are widely distributed in nature and can play a critical role in the transmission of resistance genes among species. Conventional PCR was used to amplify variable regions (i.e. 5' and 3' conserved segment) of class 1 and 2 integrons. In addition, direct amplification of integrase 1 and 2, RFLP, and sequencing were also utilized to confirm the general structure of class 1 and 2 integrons and to identify their gene cassettes. Outcome measurements include the

proportion of class 1 and 2 integrons among single drug resistance (SDR), MDR, and susceptible phenotypes (NR). Furthermore, molecular typing of the gene cassette carried by class 1 and 2 integrons was determined by RFLP. The impact of the gene cassette on antimicrobial resistance, which was carried by class 1 and 2 integrons, was further determined using curing experiments. This study is designed to help us understand the potential role of different factors into the emergence of MDR phenotype and can be more helpful in prevention and treatment of infectious disease.

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CHAPTER II

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF CLINICAL *ESCHERICHIA COLI* ISOLATES EXPRESSING MULTIDRUG RESISTANCE RECOVERED FROM CANINE AND FELINE WITH SPONTANEOUS DISEASE

Abstract

Background: Antimicrobial resistance is emerging among canine and feline clinical *E. coli* isolates. Objectives: To generate baseline data on the prevalence and molecular basis of antimicrobial resistance in the canine and feline clinical *Escherichia coli* isolates. Animals: *E. coli* pathogens isolated from dogs and cats. Methods: A total of 377 *E. coli* isolates were collected from dogs or cats with spontaneous infection between May and September 2005. Isolates were phenotypically characterized based on the susceptibility to seven antimicrobial agents by E-test® according to CLSI guidelines. Selected isolates from each phenotype were genotyped using pulsed-field gel electrophoresis (PFGE).

Results: Of the 377 isolates, 43 phenotypes were generated. Almost 44% (n= 85/194) of the resistance isolates expressed single drug resistance, with resistance to beta-lactams predominating (83%; n=71/85). Only two isolates expressed single drug resistance to

enrofloxacin. Whereas 56% (n= 109/194) of the resistant isolates expressed multidrug resistance, with twenty isolates were resistant to all drugs. The drugs most commonly involved in multidrug resistance were amoxicillin (96.3 %; 105/109), amoxicillin-clavulanic acid (85%; 93/109) and enrofloxacin (61.5%; 67/109). Among all four regions, multidrug resistance tended to be greater in the South region (67.9%; 57/84) and least in the West, (46.6 %, 14/30). Dendrograms for 34 *E. coli* isolate from the state of Alabama revealed a total of 26 different PFGE profiles (based on ≥ 90% similarity), representing 12 different phenotypes.

Conclusions: These results indicated that phenotypes and genotypes are not related, and that pathogenic *E. coli* have an extensive genetic diversity. The South had a greater incidence of multidrug resistance *E. coli*.

Introduction

The emergence of antibiotic resistance has been documented in *E. coli* isolates from human, animal and environmental sources (von Baum and Marre, 1995). The MDR *E. coli* is an emerging condition among clinical samples from small animals (Normand *et al.,* 2000). Several studies have demonstrated an increased in multi-drug resistance *E. coli* as a cause of infection in dogs and cats both in the United State and Europe (Cohn *et al.,* 2003; Guardabassi *et al.,* 2004; Normand *et al.,* 2000a, 2000b). Previous work by the authors indicated a high incidence of MDR in *E. coli* isolates associated with first time urinary tract infection (UTI) from canine patients admitted to Auburn University's Veterinary Teaching Hospital (n=175). Overall *E. coli* resistance to all 17 antimicrobials

was 31% and approximated 40% to 50% toward "first choice" empirical antimicrobials (amoxicillin, amoxicillin-clavulanic acid, cephalexin and sulfonamide-trimethoprim) and toward enrofloxacin. Those factors associated with a greater percent resistance ($p \geq$ 0.0005) were gender (males), previous antimicrobial therapy (within 30 days), immune suppressive drugs (within 30 days), and days of hospitalization ($\geq$ 5days)[a]. Nosocomial infection associated with multi-resistant *E. coli* has been reported in dogs in intensive care units, and extensive use of broad-spectrum antimicrobials is a contributing factor for multi-drug resistance (Sanchez *et al.,* 2002).

In addition to the impact on animal health, emergent pathogenic multi-resistant *E. coli* may have significant public health consequences if isolates are transmitted to humans from their pets (Beutin, 1999; Johnson *et al.,* 2001). For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) isolates recovered from pets closely resembled MRSA human isolates which are widespread in German hospitals (Strommenger *et al*., 2006). Thus, a possibility exists of cross-transmission of MRSA from pets to humans (Strommenger *et al*., 2006). A similar concern is now emerging with *E. coli* clinical isolates. Phylogenetic and pathotypic similarities between *E. coli* isolates from urinary tract infections in dogs and extraintestinal pathogenic *E. coli* (ExPEC) infections in humans have been described (Johnson *et al.,* 2001; Starcic, *et al.,* 2002). Over 15% of canine feces were found to contain *E. coli* strains closely related to human virulent ExPEC clones (Johnson *et al.,* 2001).

Molecular characterization of resistant isolates utilizing fingerprinting and hybridization techniques, such as pulsed-field gel electrophoresis (PFGE), polymerase

chain reaction (PCR) and genetic sequencing, are important tools to identify the spread of clonal unites. Among these methods, genetic fingerprinting of strains using PFGE is particularly useful for demonstrating close relationships among strains, including those manifesting MDR.

Understanding the patterns of antimicrobial resistance in canine and feline *E. coli* isolates is important not only from a veterinary medicine prospective, but also from a global public health prospective. The multi-drug resistant bacteria are detected with an increasing frequency in populations exposed to antimicrobial gents. Therefore, it will become increasingly important for a veterinarian to understand the impact of antimicrobial agents and the emergence of antimicrobial resistance patterns. In this study, we extend the evaluation and monitoring of the emerging resistances and molecular bases of antimicrobial resistance among *E. coli* isolates in the U.S. In the present study, canine and feline isolates of *E. coli* with spontaneous infections were collected from different regions in the US and were characterized phenotypically using the susceptibility testing and genotypically using PFGE standard protocol.

Materials and methods

Sample collection and handling

Isolates (n = 377) of *E. coli* were collected from dogs or cats with spontaneous infection between May and September 2005. The isolates were collected from the different tissue sites. Those isolates were previously isolated and cultured from samples submitted by veterinary practitioners to commercial or veterinary teaching hospital

microbiology laboratories serving veterinary practices throughout the US. Cultured isolates were then submitted by nine microbiology (commercial and academic) laboratories in the US to the Clinical Pharmacology Laboratory at Auburn University. Laboratories were divided, based on proximity, into four geographical areas: South, comprising the states of Alabama (AU), Mississippi (MSU), and North Carolina (NCSU); West, with the states of Washington (WSU), and California (IDC), Midwest, including Indiana (CL), Kansas (KSU), and Wisconsin (MAR), and Northeast, with the state of Massachusetts (IDM). Collaborating laboratories submitted isolates on trypticase agar slants (shipped overnight at room temperature). Upon receipt, isolates were streaked on trypticase agar and incubated at 37^{o}C for 24 h. For standard susceptibility testing, samples were adjusted to 0.5 McFarland standard turbidity (~10^{8} CFU) and a sterile swab was used to inoculate two 150 mm Mueller Hinton plates (MH).

Susceptibility testing

Susceptibility to 7 drugs, representing 5 classes of antimicrobials, was determined using the E-test®[b] according to Clinical and Laboratory Standards Institute (CLSI; previously the National Committee on Clinical Laboratory Standards) guidelines and interpretive standards (CLSI, 2008). Drugs tested were three beta lactams, amoxicillin (A), amoxicillin-clavulanic acid (X), and cefpodoxime (P); a tetracycline, doxycycline (D); a fluoroquinolone, enrofloxacin (E); an aminoglycoside, gentamicin (G); and a "potentiated" sulfonamide, trimethoprim-sulfamethoxazole (T). The drugs were selected based on different mechanisms of action and their historical use on veterinary medicine.

MH plates were incubated at 35°C for 20-24 h. The MIC results were recorded from the strips. Each isolate was then designated as resistant (R) or susceptible (S) to each drug. The CLSI intermediate designation (I) was treated as R using CLSI guidelines and interpretive standards (Livermore *et al.,* 2002). For control purposes, *E. coli* ATCC 25922[c] (range MIC = 0.016-0.125µ/mL) and *S. pneumoniae* ATCC 49619[c] (range MIC = 0.5-2.0µ/mL) were used.

Phenotypic characterization

Phenotypes were generated for each isolate and categorized as no resistance (NR), resistant to a single drug or drug class (SDR) (A, X and P considered a drug class) or resistant to two or more drug class (MDR). The distribution of each phenotype expressed for each region or each tissue source was determined.

Statistical analysis

The associations between the various categories, including the geographical and tissue factors, and the emergence of MDR versus SDR phenotypes, were assessed using the chi square test (X^2) using MINITAB® 15 package.[d] *P* values of < 0.05 were reported as statistically significant.

Genotypic characterization

For genotyping, at least two isolates of each phenotype were selected from each region for PFGE (for a total 90 isolates). PGFE was performed according to protocols

developed by the Centers for Disease Control and Prevention (Atlanta, GA) (Barrett *et al.,* 1994). Briefly, the genomic DNA of each isolate was embedded inagarose plugs and the plugs were transferred to 50-ml tubes[e] containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosine, 0.1 mg of proteinase K/ml). Samples were lysed for 120 min at 54°C in an orbital shaker water bath with constant agitation (150 to 200 rpm). A total of six washes (twice with sterile ultrapure water and four times with 0.01 M Tris-EDTA buffer, pH 8.0) were used to remove excess reagents and cell debris from the lysed samples. Samples were restricted with *XbaI*[f] (40 to 50 U of enzyme at 37°C for 2 h). The plugs were loaded onto a 1% agarose-Tris buffer gel[g], and PFGE was performed with a CHEF Mapper XA apparatus[h]. DNA was electrophoresed for 18 h at a constant voltage of 200 V (6 V/cm), with a pulse time of 5.0 to 40.0 s, an electric field angle of 120[o], and a temperature of 14°C, before being stained with ethidium bromide.

Computer analysis of PFGE patterns

Gel images were captured on a Gel Doc imaging system[i]. The images were converted to a TIFF file using the automated GeneSnap[j], and analyzed with BioNumerics software[k]. The TIFF images were normalized using three standards per gel (*Salmonella* serotype Braenderup H9812), which were loaded on lanes 1, 7, and 15 (Hunter *et al*., 2005). Matching and dendrogram (UPGMA) analysis of the PFGE patterns was performed using the Dice correlation coefficient.

Results

Sample descriptions and antimicrobial susceptibility

There were 308 samples collected from dogs and 69 collected from cats. The majority of isolates were submitted by the South region (n=133), while the West region submitted 79 isolates (Table 1). Isolates from the South region had the highest percentage of resistance, while the West region had the least percent of resistance compared with other regions. The ratio of the different phenotypes varied among regions (Table 1). However in the South region, the phenotype Z (resistant to all seven antimicrobials) had the highest prevalence among phenotypes (28%, n=16/57). Resistant isolates were most recovered from urine samples in all regions. For example, urine sample represented 61.4% (n= 51/80) and 54.7% (n=29/53) in the South and Midwest regions respectively (Table 2). Among tissue sites, the soft tissue had the highest percent of resistant isolates (73.3 %, n=11/15) while the ear tissue had the least percent of resistance (29%, 8/27) compared with other tissues (Fig 1). Similarly, all phenotypes were distributed in different proportions among different tissues with no particular pattern observed.

Phenotypic characterization

The result of antimicrobial susceptibility of *E. coli* (*n* = 377) to 7 antimicrobials used in veterinary medicine was evaluated (Fig 2A). Forty-four different phenotypes (including no resistance [NR] and resistance to all tested drugs [Z]) occurred among the 377 *E. coli* isolates. Of the resistant isolates, 85 were grouped within eight SDR phenotypes, whereas 109 isolates grouped within 35 MDR phenotypes. Among the SDR

phenotypes, beta lactams were the most commonly represented class of drugs to which resistance was encountered (83.5%, n=71/85) and this included amoxicillin (30.5%, n=26/85) and amoxicillin-clauvalanic acid (28.2%, n=24/85). Other strains were resistant to combinations of amoxicillin, amoxicillin-clauvalanic acid and cefpodoxime (23.5%, n=20/85). Furthermore, less than 20% (n=14/85) of the isolates were resistant to gentamicin, cefpodoxime, doxycycline, enrofloxacin, or trimethoprim-sulfamethoxazole (Fig 2A).

The most common MDR phenotypes was Z and the least common was the phenotype resistant to only 2 drug classes (Fig 2B). The percent resistant to 5, 6 or all 7 drugs were 16.6% (n=16), 21.1% (n=23) and 18.3% (n=20), respectively (Fig 2B). The remaining MDR were represented by 34 different phenotypes. Amoxicillin was the drug that was mostly associated with MDR phenotypes 96.3 %; 105/109), followed by amoxicillin-clauvalanic acid (85%; 93/109), and enrofloxacin (61.5%; 67/109).

Effects of regions and tissue sites on microbial susceptibility

Among the four different regions, the South region had the highest percentage of MDR phenotype (67.9%, n=57/84), while the West region had the highest percentage of susceptible phenotype (62%, n=49/79) (Fig 3). No relationship was identified ($p = 0.066$) between regions and the emergence of SDR versus MDR phenotypes. The tissue exhibiting the highest proportion of MDR was skin (75%, n=12/16). In contrast, ear tissue showed the high proportion of susceptible phenotypes (70%, n=19/27) (Fig 4). However, no evidence exists for the association ($p = 0.664$) between tissues and the

presence of SDR and MDR phenotypes. The removal of tissues with low isolate numbers from the analysis did not influence the final results.

PFGE and genotypic characterizations

A total of 90 *E. coli* isolated from dogs and cats with different phenotypes yielded a total of 82 different PFGE patterns. Samples with similarity coefficients of 90–100% were considered identical. In the South, the largest proportion of resistant isolates were submitted by Auburn University (n=32) compared with Mississippi (n=26) and North Carolina (n=27). Among the selected isolates (n=34; 12 phenotypes), a total of 26 different PFGE patterns were identified (Fig 5). Thus phenotypes were not genotypically related as indicated by the different PFGE profiles of the SDR phenotypes: (AX) and (AXP). One large profile of six isolates and three different phenotypes were genetically related. In addition, three pairs with identical profiles were identified. Within each pair or cluster only isolates with distinct phenotype were found. For the tissue samples, the majority of clinical *E .coli* isolates were from urine. Among the 46 uropathogens, a total 43 different PFGE patterns were identified (based on 90% similarity level). This represents a total of 13 different phenotypes. Only two profiles from Auburn University, one of 3 isolates and the other with 2 isolates, were related, each phenotypically distinct (Fig 6).

Discussion

Our current study was confined with restricted time frame (summer 2005), limited sample size, retrospective design, and potential antimicrobials selection bias (limited to

seven antimicrobial agents). However, we studied a broad geographic collection of isolates and a different representation of tissue samples. Furthermore, we utilized E test as method of antimicrobial susceptibility testing because at it can provide a wide range of MIC lower and higher than MIC break point.

The emergence of antimicrobial resistance among pathogens in human or in animal medicine draws attention towards the increase in the risk of therapeutic failure (Costa *et al.,* 2007). In the present study, 56.2% (n=109/194) of *E. coli* were phenotypically resistant to multiple antimicrobials (Fig 2B). The majority of *E. coli* isolates were distinct based on the analysis of PFGE fingerprint patterns. Our results indicated that there is no correlation between the phenotype and the genotypes within the same region (Fig 5), or within the same tissue (i.e. urine sample) (Fig 6). This observation is in agreement with studies showing that the antibiotic resistance patterns differed significantly within PFGE strain types among uropathogenic *E. coli* isolated from dogs (Cooke *et al.,* 2002; Drazenovich *et al.,* 2004; Mentula *et al.,* 2006). The possibility of lateral horizontal transfer of resistance mobile genetic elements, such as plasmids, integrons or phage-mediated exchange, could play a role in the difference of PFGE patterns in our study as previously reported (Cooke *et al.,* 2002; Drazenovich *et al.,* 2004; Mentula *et al.,* 2006). The different profiles on PFGE of *E. coli* can be also explained by the large genomic diversity among *E. coli.* This diversity has also been demonstrated by early studies using multilocus enzyme electrophoresis (Milkman, 1973, 1997), and we know now that multiple isolates considered identical clones and groups of isolates that have similar clonal complexes are frequently recovered from diverse

geographical and temporal origins (Feil and Spratt, 2001). In the case of medically important but numerically small number of pathogenic lineages of *E. coli*, such as enteropathogenic or enterohaemorrhagic strains, recombination appears to play a limited role on the genetic diversity. But the contribution of recombination to the genetic variability of *E. coli* isolates that produce animal disease has not been addressed (Feil and Spratt, 2001). Many antimicrobials used to treat infections in dogs and cats tend to increase the prevalence of antimicrobial resistance (von Baum and Marre, 1995). The present study revealed a high percentage of resistance to the beta lactams class (i.e. amoxicillin, amoxicillin-clauvalanic acid), a class that is also associated with MDR phenotypes (Fig 2A, 2B). Similarly, the trend of increasing resistance among canine and feline *E. coli* isolates in the UK during the years 1989–1997 was toward amoxicillin-clavulanic acid and streptomycin, with many isolates showing multi-drug resistance phenotypes (Normand *et al.,* 2000a). The same observations were found among nonenteric *E. coli* isolates from dogs in the US between 1990 and 1998 , in which isolates exhibited resistance to amoxicillin, carbenicillin, and cephalothin (Oluoch *et al.,* 2001). Furthermore, Feria *et al.* (2002) characterized the pattern and mechanism of β-lactams resistance among 72 uropathogenic *E. coli* isolated from dogs, and illustrated that 36% of the isolates exhibited resistance to amoxicillin and 19% of isolates were resistance to amoxicillin-clavulanic acid**.**

The mechanism of hyperproduction of the plasmid encoded TEM-1 ß-lactamase or chromosomally encoded AmpC ß-lactamase could contribute to the emergence SDR phenotype as previously described (Bradford *et al.,* 1999; Feria *et al.,* 2002). On the other

hand, the third generation cephalosporins (i.e. cefpodoxime) have a broad spectrum activity and were approved for treating acute and chronic skin infections in dogs (Cherni *et al.,* 2006). Only one out of 194 *E. coli* isolates from dogs in this study was resistant to cefpodoxime. This observation is consistent with the observations by Feria *et al.* (2002) who found that resistance was more often to cephalothin (25%) than to either ceftazidime or ceftriaxone (1.4%).

Our observations indicated that fluoroquinolone-resistant isolates (i.e. enrofloxacin) were more likely to be multi-drug resistant. Only 2 out 85 isolates expressed SDR phenotype, while 69 out 71 isolates were MDR. Enrofloxacin-MDR isolates showing resistance towards a β-lactam antimicrobial agent have the highest prevalence of the association compared to other drug classes (Fig 7). Specifically, enrofloxacin isolates which are MDR phenotype have the highest prevalence of resistance toward A (97%), followed by AX (94%). The enrofloxacin-MDR phenotype was present in about half of the isolates with resistance to T (63%), P (60%), G (53%), and D (53%). This observation is in agreement with previous reported studies which also suggest that the selection of fluoroquinolone-resistant mutants of *E. coli* is more frequent. Cooke *et al.* (2002) indicated that the enrofloxacin resistant isolates were also MDR to at least three other antimicrobials that are commonly used to treat UTI. Similarly, studies from human medicine indicated the association of fluoroquinolone with multidrug-resistant phenotypes commonly with the clinical cases of uropathogenic *E. coli* (Karlowsky *et al.,* 2002, 2003, 2006). The appearance of multidrug-resistant phenotypes maybe correlated with the higher use of fluoroquinolones in veterinary medicine as

reported in human medicine (Karlowsky *et al.,* 2006), along with inadequate infection hygiene, and sanitation control measures (Johnson *et al.,* 2006).

The present study showed an increased emergence of multidrug-resistant isolates which is consistent with previously published studies. For example, Sanchez *et al.* (2002) characterized 34 of multidrug-Resistant *E. coli* isolates associated with nosocomial infections in dogs. The isolates were resistant to most cephalosporins, beta-lactams, and the beta-lactamase inhibitor clavulanic acid as well as resistance to tetracycline, spectinomycin, sulfonamides, chloramphenicol, and gentamicin. In our study, the MDR phenotypes were associated with the South region (Fig 3). In contrast, the West region had the highest percent of susceptible phenotype with lowest percent of MDR phenotype. These results probably highlight the different prescribing practices by the veterinarians among different geographic regions. In other words, increasing prescription of the broad spectrum antimicrobials (i.e. fluoroquinolones) at veterinary hospitals and clinics may be contributing to this observation, as previously reported (Sanchez *et al.,* 2002). There is a possibility that more antimicrobial agents were prescribed based on the veterinarian's first choice and neither was based on antimicrobial susceptibility testing or considered the previous antimicrobial case history. Focusing on the previous antimicrobial drug used and their resistance mechanisms of the pathogen will be determined for our next studies.

The dissemination of MDR phynotypes among *E. coli* isolates from skin tissue may occur as a consequence of horizontal gene transfer (Osborn and Böltner, 2002). Recent evidence indicates that aerobic Gram-positive corynebacteria can act as reservoir of class 1 integrons in poultry litter (Nandi *et al.,* 2004). This indicates that the

environment could play critical role in genes cassette exchange between different mobile integrons. With increase sequence information, many resistance genes have been found in many bacterial species, and homologous genes share high similarities among non-related bacteria (Aarestrup, 2006). The situation becomes very important in the exposed environment, such as the skin, in which the spread of resistance gene can be facilitated among heterogeneous bacterial communities through some mobile elements (Dionisio *et al.,* 2002).

In conclusion, the antimicrobial-susceptible and multidrug-resistant phenotypes identified in this study were genetically not related by PFGE within the same tissue or among the same geographic location collected over the same period. Furthermore, this study indicated large genomic diversity among clinical *E. coli* isolated from dogs and cats from different regions and/or tissues. MDR phenotypes were associated with South regions and enrofloxacin resistance was associated with MDR phenotypes. Therefore, a large surveillance study in which more *E. coli* isolates from different regions with previous antimicrobial history will be conducted in our laboratory. This will help a veterinarian to understand the impact of previous antimicrobials use on the emergence of *E. coli* MDR phenotypes. Additionally, class 1 integrons were established to be more common in clinical *E. coli* isolates from animals and further work is going on in our laboratory to characterize these integrons.

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Regions[a]	No. of resistant phenotype(s)[b]	Antimicrobial phenotype(s)[c]
Midwest (108)	53 (49 %)	A (10); AD, AXDEGT, AXET, AXP, AXPD, AXPDET, E (2); ADG, AT, AXD, AXDE, AXDET, AXE, AXEGT, AXPDEG, AXPDT, AXPEGT, AXPET, AXPGT, AXT, DE, DEG, P, T, Z (1); AX (6); D (5)
Northeast (57)	27 (47.3 %)	A, AX (4); ADEGT, AXDGT, AXDT, AXE, AXPDEG, AXT, E (1); AXDEGT, AXPD (2); AXP (5); Z (3)
South (133)	83 (62.4 %)	A, AXPE (5); AD, ADET, ADGT, AP, AXD, AXDET, AXDGT, AXDT, AXET, AXPDGT, AXPDT, AXPEG, AXPEGT, AXPT, AXT, T (1); AT, AXE, AXDEG, AXEGT, AXEGT, AXPD, AXPDEG, D (2); AX, AXP (9); AXPDET (8); Z (16)
West (79)	30 (38 %)	A (6); AD, AT, AXPDE, AXPDEG, AXPEGT, AXT, D, DT, EGT (1); AX (5); AXD, AXPD (3); AXP (4)

Table 1. Distribution of resistant phenotypes and their geographical locations.

A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

[a] Number in parentheses is the total number of isolates collected from that region.

[b] Number in parentheses is the percent of resistant isolates collected from that region.

[c] Number in parentheses is the total number of phenotype(s) collected from that region.

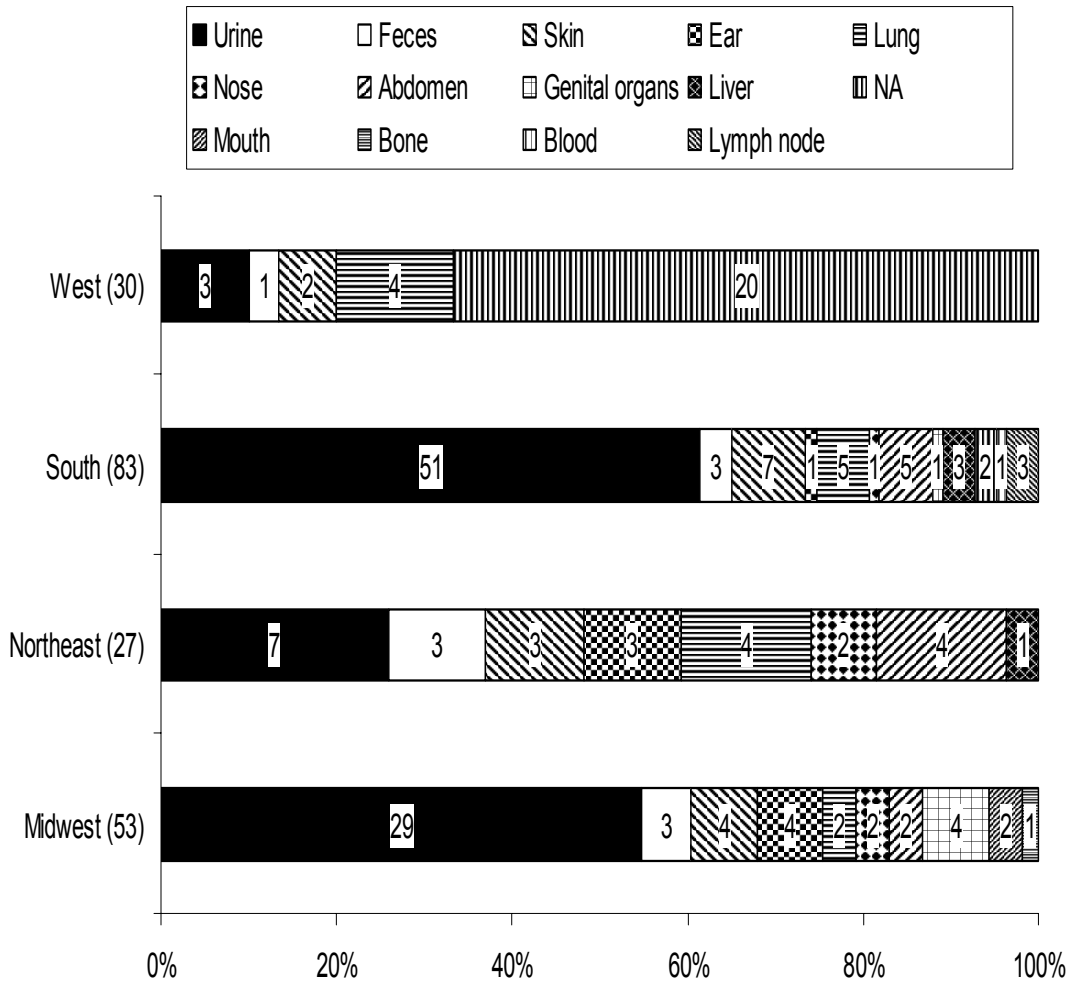

Figure 1. Resistant isolates of *E. coli* from four US regions and their clinical sources.

NA = the source was not determined.

[a] Number in parentheses is the total number of isolates collected from that region.

[b] Number in parentheses is the percent of resistant isolates collected from that region.

[c] Number in parentheses is the total number of resistant isolates collected from the tissue site.

Type of infection[a]	No. of phenotype(s)[b]	Antimicrobial phenotype(s)[c]
Skin (27)	16 (59.2 %)	A, AX, AXE, AD, AXPD, AXDGT, AXPDET, ADET, AXPEGT (1); Z (3); AXDEGT, AXP (2)
Ear (27)	8 (29.6 %)	A, AX, (2); AXPDEG, AXT, AXE, Z (1)
Lung (22)	15 (68.2 %)	A, P, AX, AXDET, AXPT, AXPE, AXPD (1); Z, AXP (3); AXT (2)
Soft Tissue (15)	11 (73.3 %)	A, AXPDET, T, AXPT, AXD, AXDT, AXP, AXPDET, AX (1); Z (2)
Urine (174)	91 (52.3 %)	A (12); AXDET, AT, AXPEGT, AXPDT, AXET, AXDET, AXPDEG (2); AXPDET (7); AXP (8); AXPE, AD, E, AXPD, AXEGT (3); AXDT, T, AXDGT, AXT, ADGT, AP, DE, DEG, AXPDE, AXD, AXE (1); D (6); AX, Z (9)

Table 2. Distribution of resistant phenotypes with their clinical sources.

A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

[a] Number in parentheses is the total number of isolates collected from that tissue.

[b] Number in parentheses is the percent of resistant isolates collected from that tissue.

[c] Number in parentheses is the total number of R phenotype(s) collected from that tissue.

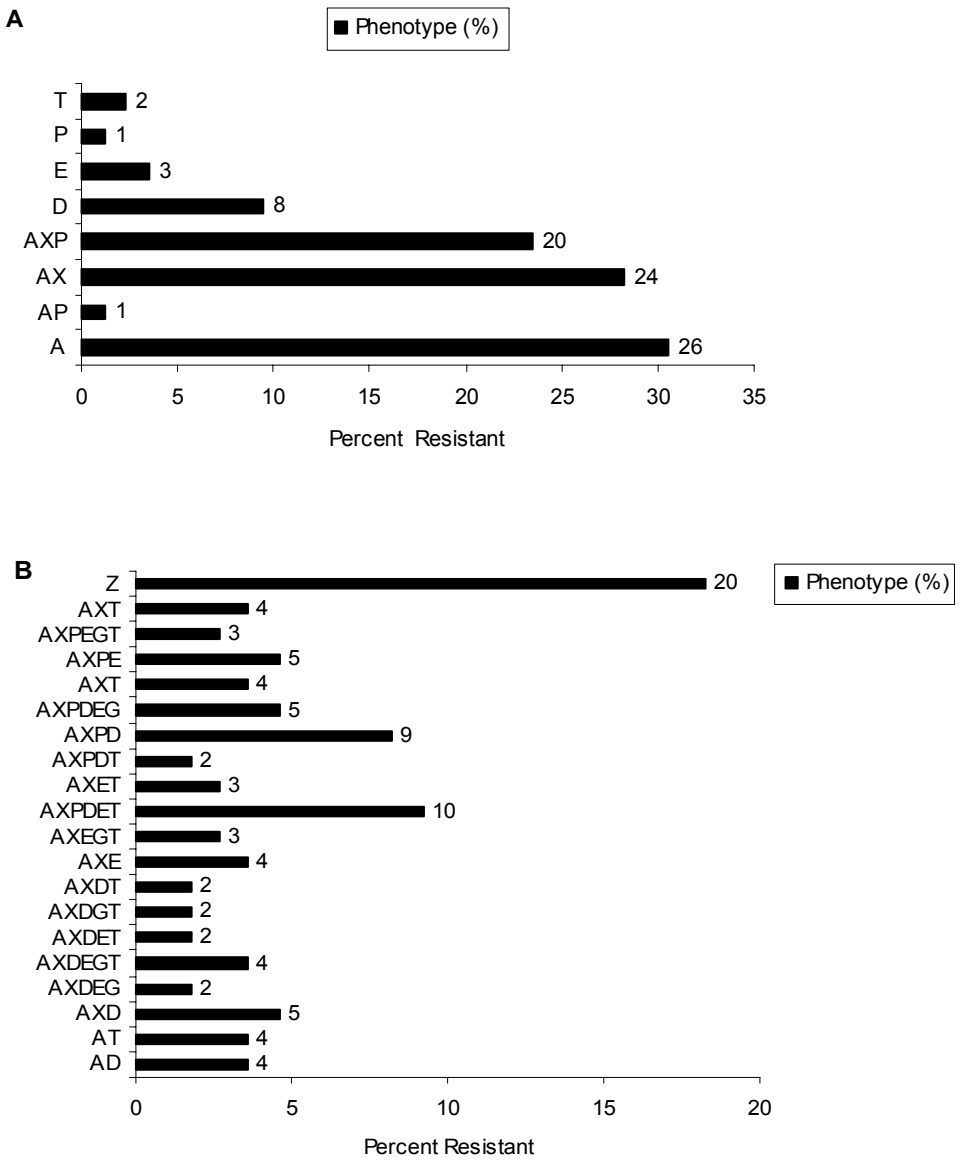

Figure 2. The number and proportion (%) of each SDR (A) and MDR (B) phenotype(s) expressed in 377 isolates of *E. coli* causing spontaneous disease in dogs and cats throughout the US. Numbers of isolates are indicated above each bar.

SDR = single drug resistance; MDR = multiple drug resistance; N = susceptible phenotype. Z = resistance to all 7 drugs. A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

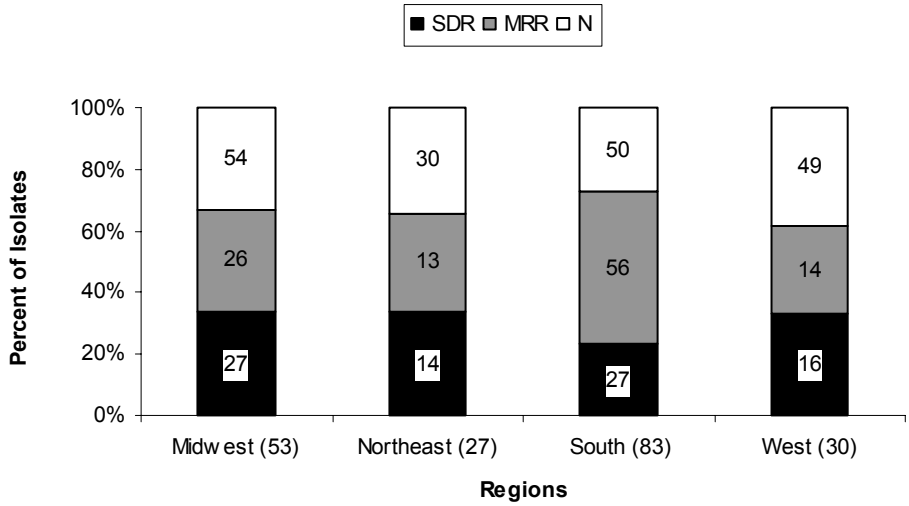

Figure 3. The overall proportion of SDR, MDR, and NR phenotypes among *E. coli* clinical isolates by geographical region. For SDR and MDR, the proportion is of total resistant isolates (n=194); for NR, the proportion is out of all 377 isolates.

Numbers of isolates for each phenotype are indicated within each bar.

SDR = single drug resistance; MDR = multiple drug resistance; N = susceptible phenotype.

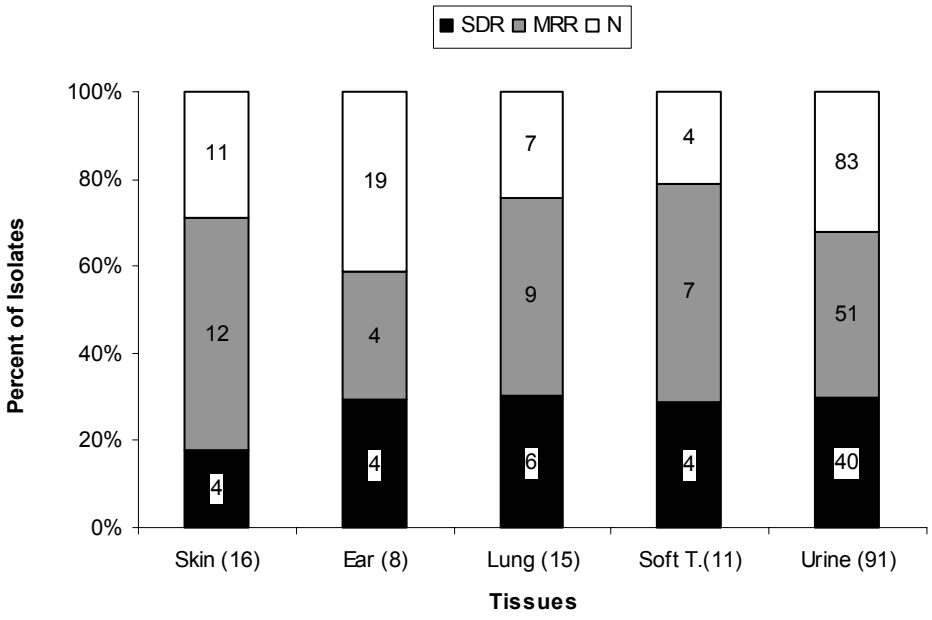

Figure 4. The overall proportion of SDR, MDR, and NR phenotypes among *E. coli* clinical isolates by tissue source. For SDR and MDR, the proportion is of total resistant isolates (n=194); for NR, the proportion is out of the total isolates in that tissue.

Numbers of isolates for each phenotype are indicated within each bar.

SDR = single drug resistance; MDR = multiple drug resistance; N = susceptible phenotype.

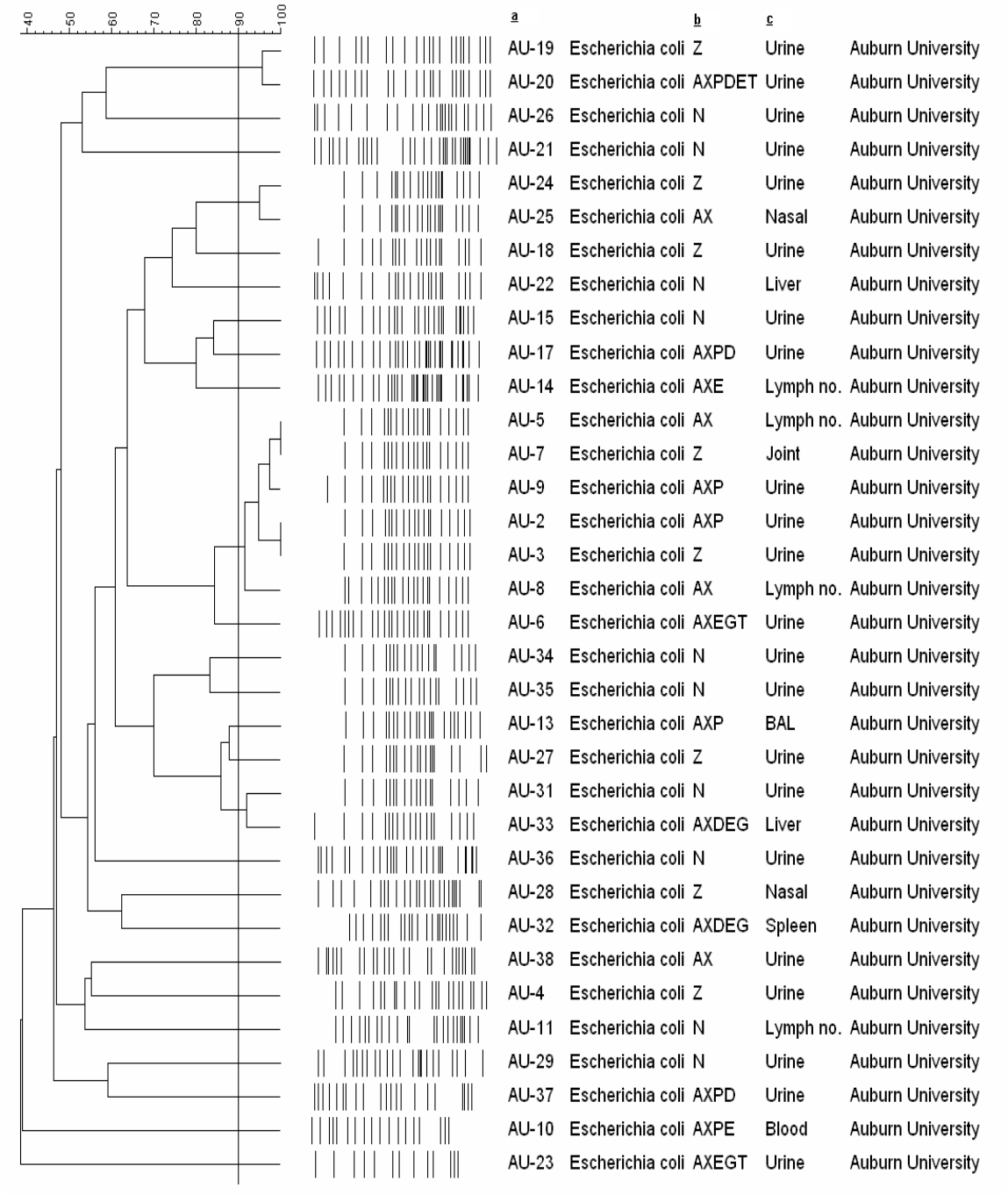
Dice (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
40
50
60
70
80
90
100
a
b
c
AU-19 Escherichia coli Z Urine Auburn University
AU-20 Escherichia coli AXPDET Urine Auburn University
AU-26 Escherichia coli N Urine Auburn University
AU-21 Escherichia coli N Urine Auburn University
AU-24 Escherichia coli Z Urine Auburn University
AU-25 Escherichia coli AX Nasal Auburn University
AU-18 Escherichia coli Z Urine Auburn University
AU-22 Escherichia coli N Liver Auburn University
AU-15 Escherichia coli N Urine Auburn University
AU-17 Escherichia coli AXPD Urine Auburn University
AU-14 Escherichia coli AXE Lymph no. Auburn University
AU-5 Escherichia coli AX Lymph no. Auburn University
AU-7 Escherichia coli Z Joint Auburn University
AU-9 Escherichia coli AXP Urine Auburn University
AU-2 Escherichia coli AXP Urine Auburn University
AU-3 Escherichia coli Z Urine Auburn University
AU-8 Escherichia coli AX Lymph no. Auburn University
AU-6 Escherichia coli AXEGT Urine Auburn University
AU-34 Escherichia coli N Urine Auburn University
AU-35 Escherichia coli N Urine Auburn University
AU-13 Escherichia coli AXP BAL Auburn University
AU-27 Escherichia coli Z Urine Auburn University
AU-31 Escherichia coli N Urine Auburn University
AU-33 Escherichia coli AXDEG Liver Auburn University
AU-36 Escherichia coli N Urine Auburn University
AU-28 Escherichia coli Z Nasal Auburn University
AU-32 Escherichia coli AXDEG Spleen Auburn University
AU-38 Escherichia coli AX Urine Auburn University
AU-4 Escherichia coli Z Urine Auburn University
AU-11 Escherichia coli N Lymph no. Auburn University
AU-29 Escherichia coli N Urine Auburn University
AU-37 Escherichia coli AXPD Urine Auburn University
AU-10 Escherichia coli AXPE Blood Auburn University
AU-23 Escherichia coli AXEGT Urine Auburn University

Figure 5. Dendrogram of SDR, MDR, and N phenotypes among *E. coli* clinical isolates collected at the same institution (South).

N = susceptible phenotype; Z = resistance to all 7 drugs. A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

[a] Isolates' identification.

[b] The phenotype for each isolate.

[c] Isolates' tissue source.

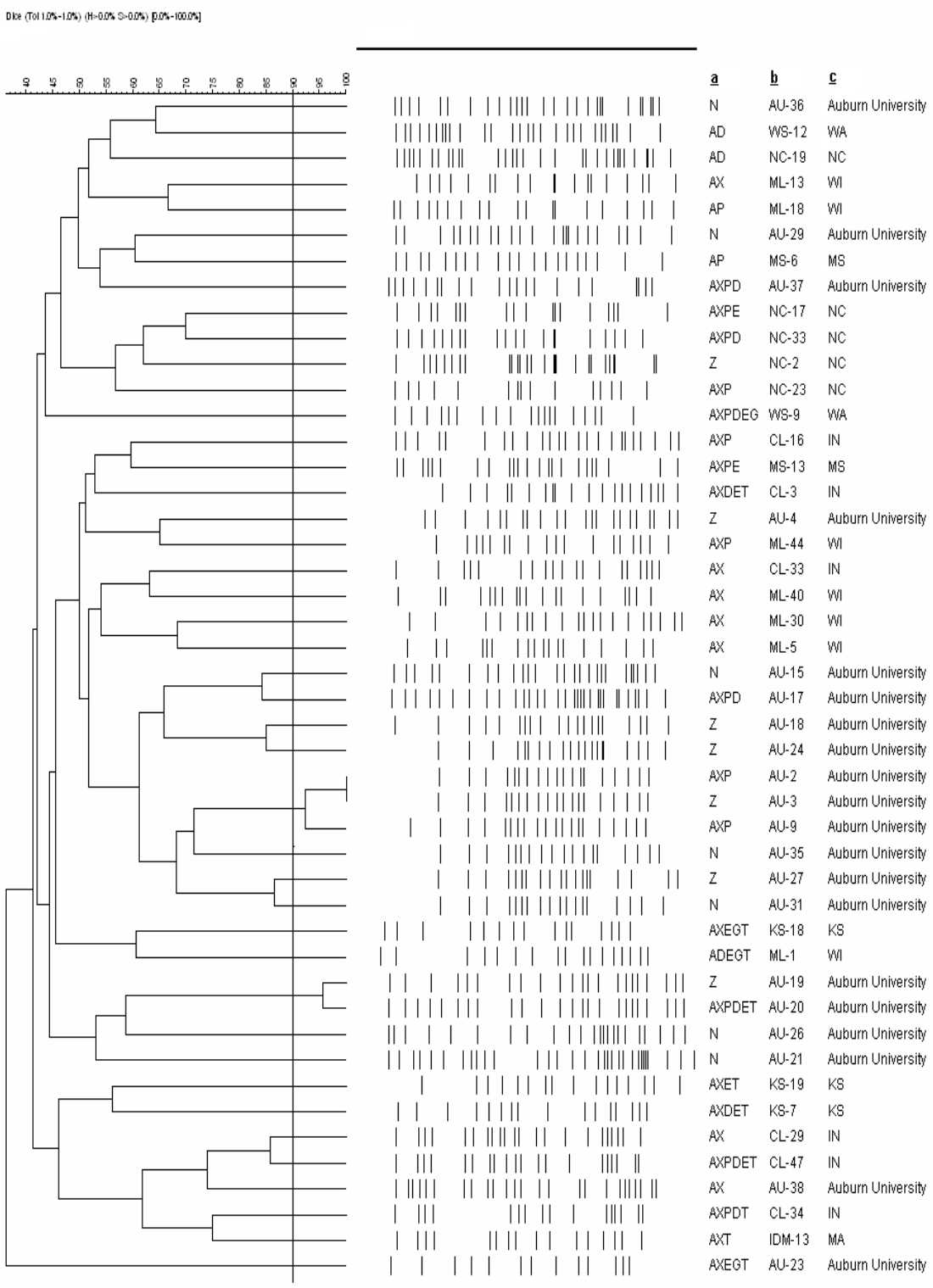
Dice (Tol 1.0%-1.0%) (H=0.0% S=0.0%) [0.0%-100.0%]
40 45 50 55 60 65 70 75 80 85 90 95 100
a b c
N AU-36 Auburn University
AD WS-12 WA
AD NC-19 NC
AX ML-13 WI
AP ML-18 WI
N AU-29 Auburn University
AP MS-6 MS
AXPD AU-37 Auburn University
AXPE NC-17 NC
AXPD NC-33 NC
Z NC-2 NC
AXP NC-23 NC
AXPDEG WS-9 WA
AXP CL-16 IN
AXPE MS-13 MS
AXDET CL-3 IN
Z AU-4 Auburn University
AXP ML-44 WI
AX CL-33 IN
AX ML-40 WI
AX ML-30 WI
AX ML-5 WI
N AU-15 Auburn University
AXPD AU-17 Auburn University
Z AU-18 Auburn University
Z AU-24 Auburn University
AXP AU-2 Auburn University
Z AU-3 Auburn University
AXP AU-9 Auburn University
N AU-35 Auburn University
Z AU-27 Auburn University
N AU-31 Auburn University
AXEGT KS-18 KS
ADEGT ML-1 WI
Z AU-19 Auburn University
AXPDET AU-20 Auburn University
N AU-26 Auburn University
N AU-21 Auburn University
AXET KS-19 KS
AXDET KS-7 KS
AX CL-29 IN
AXPDET CL-47 IN
AX AU-38 Auburn University
AXPDT CL-34 IN
AXT IDM-13 MA
AXEGT AU-23 Auburn University

Figure 6. The dendrogram for *E. coli* phenotypes collected from one tissue (urine). N = susceptible phenotype; Z = resistance to all 7 drugs. A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

[a] The phenotype for each isolate

[b] Isolates' identification.

[c] The regional source of isolates.

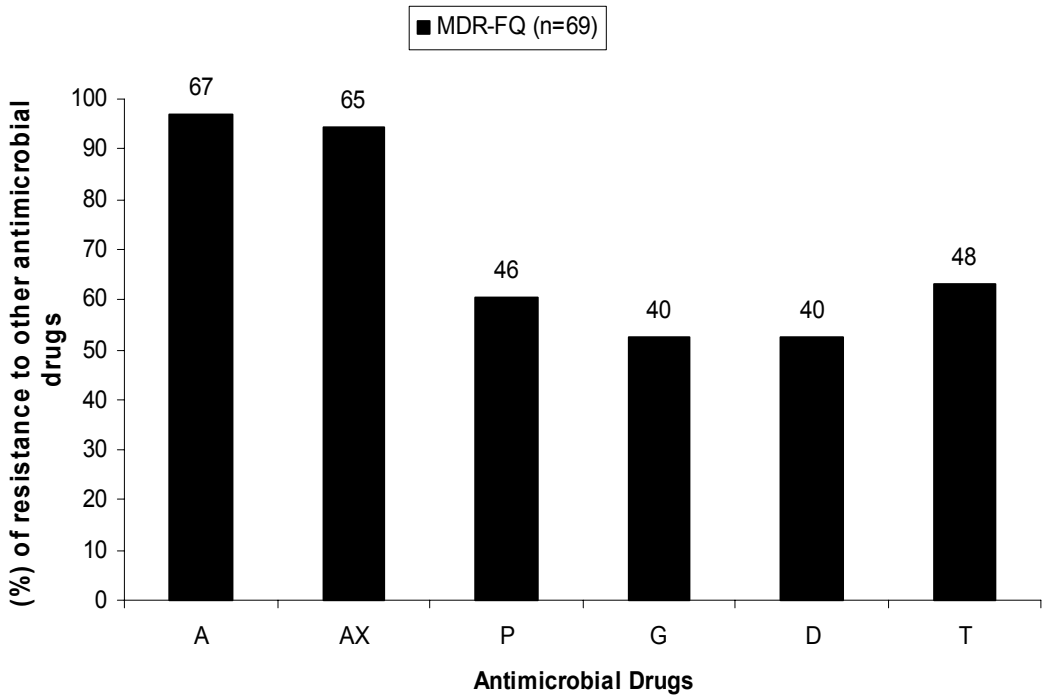

Figure 7. Frequency of the MDR phenotype in enrofloxacin isolates showing resistance to non-fluroquinolone antimicrobial agents. Numbers of isolates are indicated above each bar. A = amoxicillin; X = amoxicillin-clavulanic acid; P = cefpodoxime; D = doxycycline; E = enrofloxacin; G = gentamicin; T = trimethoprim-sulfamethoxazole.

Footnotes

[a] Boothe DM, Smaha T, Hathcock T, *et al*. *Escherichia coli* Resistance in the Canine Urinary Tract. Am Coll Vet Intern Med Ann Forum, Baltimore, MD, June 2005 (abstract).

[b] Epsilon; AB Biodisk, Solna, Sweden.

[c]American Tissue Cell Culture, Manassas, VA.

[d]Minitab 15, statistical software, State College, PA, USA.

[e]polypropylene tubes; 30 by 115 mm; Becton Dickinson, Franklin Lakes, NJ.

[f]*XbaI,* restriction enzyme; Roche Applied Science, Indianapolis, IN.

[g]SeaKem Gold Agarose; BioWhittaker Molecular Applications, Rockland, ME.

[h]CHEF Mapper XA; Bio-Rad Laboratories, Hercules, CA.

[i]Gel Doc imaging system; Syngene, Frederick, MA.

[j]GeneSnap; Syngene, Frederick, MA.

[k]BioNumerics software; Applied Maths, Sint-Martens-Latem, Belgium.

CHAPTER III

DETECTION OF FLUOROQUINOLONE RESISTANCE LEVEL IN CLINICAL CANINE AND FELINE *ESCHERICHIA COLI* PATHOGENS USING RAPID REAL-TIME PCR ASSAY

Abstract

Fluoroquinolones are used to treat infections caused by *E. coli* in canine and feline veterinary patients, particularly those infecting the urinary tract. The *gyrA* gene is a primary target causing fluoroquinolone resistance in Gram negative coliforms, with mutations in codon 83 and 87 generally associated with high level of resistance *E. coli* clinical isolates. We have developed a fluorescence resonance energy transfer (FRET) quantitative PCR to identify enrofloxacin-resistance in clinical *E. coli* isolates that carry mutations in codon 83 and 87 of *gyrA*. This real-time quantitative PCR assay is rapid, economical, and sensitive compared with cultured antimicrobial susceptibility testing. The assay identified as few as four genome copies per reaction from culture and 19 genome copies in urine. For the 70 isolates tested, the sensitivity was 87.5% (95% CI = 75% to 95.3%) (n=42/48) whereas specificity was 100% (95% CI = 87.3% to 100%) (n=22/22). Furthermore, we were able to accurately differentiate between the wild type and mutants *E. coli* directly from infected canine urine samples (n=5) within 1-2 hrs. These results were confirmed by sequence alignments of the PCR products and

comparison with the susceptibility testing. The FRET-PCR assay appears to have promising clinical application as an early diagnostic tool for rapid and sensitivedetection and differentiation of the level of fluoroquinolone resistance among clinical *E. coli*isolates.

Introduction

Escherichia coli is the major cause of urinary tract infections, pyometra and other infections in dogs and cats (Hagman and Kühn, 2002; Chen *et al*., 2003). Fluoroquinolones historically have been used in veterinary medicine to treat infections caused by *E. coli.* In recent years, a substantive increase in quinolone resistance in animal *E. coli* isolates has been reported (Cooke *et al*., 2002; Cohn *et al*., 2003; Guardabassi *et al*., 2004; Boothe *et al*., 2006). Included are studies that report an increase in the incidence of antimicrobial-resistance, including multi-drug resistant *E. coli,* particularly from isolates associated with urinary tract infections in canine patients (Hirsh, 1973; Guardabassi *et al*., 2004; Shaheen *et al*., 2008a, 2008b).

The most common mechanism of resistance to quinolones in *E. coli* are mutations in genes that encode subunits of the quinolone target topoisomerases, and specifically, DNA gyrase (topoisomerase II, in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE*) (Everett *et al*., 1996; Vila *et al*., 1996; Piddock, 1999). These mutations are primarily located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and its homologous region of the *parC* gene (Oram and Fisher, 1991; Piddock, 1999). Mutations in *gyrB* and *parE* genes are less significant and rarely contribute to quinolone resistance (Giraud *et al*., 2001; Vila *et al*., 1996). Other contributing

mechanisms of resistance include an efflux pump or modifications of porins which preclude effective intracellular drug concentrations, thus decreasing susceptibility to quinolones (Everett *et al*., 1996; Giraud *et al*., 2001). These latter mechanisms generally confer multidrug resistance (Piddock, 1999; Mazzariol *et al*., 2000).

Previously it has been reported that the predominant mechanism of high level fluoroquinolone resistance in *E. coli* appears to be a C to T transition in codon 83 and G to A, A to G, or G to T transitions in codon 87 in the QRDR of *gyrA*. The codon 83 mutation results in a Ser-83 to Leu-83 substitution whereas the codon 87 mutations results in Asp-87 to Asn-87, Asp-87 to Gly-87 or Asp-87 to Tyr-87 substitution in the functional protein (Oram and Fisher, 1991). Each of these mutations precludes binding of the drug to the target site, rendering the isolate resistant to the drug (Hooper, 1999).

Clinical and Laboratory Standards Institute (CLSI) guidelines for routine fluoroquinolone susceptibility testing include agar gel or dilution or micro-dilution methods (CLSI, 2008). For the clinician, delays of 3 to 5 days are often realized between sample acquisition and susceptibility reporting. This delay between sample collection and receipt of results is often untenable, leading the clinician to empirically treat the infection. For urinary tract infections, the infecting organisms is often (correctly) assumed to be *E. coli* (Bubenik *et al*., 2007). Drugs presumed (often incorrectly) to be effective include fluoroquinolones. In the face of increasing *E. coli* resistance to fluoroquinolones, the result is inappropriate therapy (Sanchez *et al*., 2002). Thus, there is a need for a rapid method of detecting infections associated with fluroquinolone resistant *E coli*.

A rapid PCR-based FRET method has been developed that allows detection of

ciprofloxacin resistant *Yersinia pestis* isolates that carry a mutation in codon 81 or codon 83 of *gyrA* (Lindler *et al*., 2001) and for detection of quinolone-resistant *Neisseria gonorrhoeae* in urine samples (Siedner *et al*., 2007). The basic principle of the technique is the use of two standard DNA primers. For FRET PCR, two fluorescently labeled hybridization probes bind to target DNA between the flanking primers. Hybridization probe 1 is labeled with fluorescein (the donor dye) at the 3'-end and hybridization probe 2 is labeled with LightCycler-Red 460 (LC Red 640; acceptor dye) at the 5' end. The probes hybridize to the amplified DNA fragment in a head to tail arrangement, thereby bringing the two fluorescent dyes into close proximity. The energy emitted as a result of the proximity excites the acceptor dye attached to the second hybridization probe, which then emits fluorescent light at a different wavelength. Because the stability of hybridization varies with the phenotype (i.e. wild versus mutation, and type of mutation), melting temperatures consequently vary. Discriminating among the different phenotypes that carry mutation(s) is indicated by melting curve analysis (MCA).

Most studies that focus on the mechanisms of quinolone resistance in *E. coli* have been conducted in human clinical strains. Those few reports involving animal strains generally focus on those of public health significance, that is, animals intended for food consumption (Everett *et al*., 1996; Giraud *et al*., 2001). Among these reports, most involve conventional PCR techniques, followed by sequence analysis of the amplicon. However, for our purposes, a rapid, sensitive, and specific test is needed for early identification of pathogens causing infections in clinical patients (Ko and Grant, 2006; Abdelbaqi *et al*., 2007; Siedner *et al*., 2007); if the technique can also detect the mutation level (that is, level of resistance) of the clinical *E. coli* isolates, the clinician will be

further empowered in the design of dosing regimens that support judicious antimicrobial use (Boothe, 2006). The purpose of this report is to report the detection of canine and feline clinical *E. coli* isolates that carry mutations in *gyrA* were targeted utilizing a rapid PCR-based FRET method that detects mutations in codon 83 and codon 87 in the QRDR of *gyrA*.

Materials and methods

Bacterial isolates and culture conditions

E. coli isolates used for FRET PCR assay development are listed in Table 1. *E. coli* strains ATCC 11775 (Weigel *et al*., 1998) and *E. coli* K12 (Swanberg and Wang; 1987) were used for multi-sequence alignment analysis for *gyrA* sequence. The other 70 susceptible (n= 22), intermediate (n= 5) and resistant *E. coli* isolates (n= 43) studied were acquired from clinical veterinary microbiology laboratories between May and September of 2008. In each case, the isolate had previously been cultured from a sample collected from a dog or cat with spontaneous disease by a veterinarian and submitted to the laboratory for identification and susceptibility testing. The veterinary clinical microbiology laboratory identified each isolate as *E. coli* prior to submission to the Clinical Pharmacology Laboratory (Auburn University, Auburn, AL, USA). Susceptibility testing was repeated in the Clinical Pharmacology Laboratory using a custom microtube dilution plate. Upon receipt in our laboratory, isolates were plated on CHROMagar™ Orientation (Becton, Dickinson, Franklin Lakes, NJ) for rapid confirmation and *E. coli*. isolates were streaked on trypticase agar (Becton Dickinson, Franklin Lakes, NJ) and then incubated at 35°C for 18-24 h. For standard susceptibility testing, cells were harvested and suspended in 4.5 ml of 0.9% normal saline and were

adjusted to 0.5 McFarland standard turbidity (~10^8 CFU) using SENSITITER® Nephelometer (TREK Diagnostic Systems, Cleveland, OH) before testing.

Susceptibility testing

Susceptibility to fluoroquinolone was determined for enrofloxacin (ENR) using a custom-made microdilution susceptibility testing (TREK Diagnostic Systems, Cleveland, OH) according to CLSI guidelines and interpretive standards (CLSI, 2008). Microdilution plates were incubated at 35°C for 18-24 h. The results were recorded as true value MIC using the SENSITITER® VIZION system (TREK Diagnostic Systems, Cleveland, OH). For quality control purposes *E. coli* ATCC® 25922 (American Tissue Cell Culture, Manassas, VA; [range MIC = 0.008-0.03µ/mL]) and *Enterococcus fecalis* ATCC® 29212 (American Tissue Cell Culture, Manassas, VA; [range MIC = 0.12-1.0µ/mL]) were used. Each isolate was then designated as resistant (R), intermediate (I) and susceptible (S) to enrofloxacin using CLSI guidelines and interpretive standards (CLSI, 2008). The enrofloxacin break point (BP) was defined according to CLSI guidelines as any isolates exhibition MIC = 4 µg/mL, while MIC = 0.5 µg/mL was considered to be the enrofloxacin susceptible BP (CLSI, 2008).

DNA extraction and designing of the real-time PCR

Bacterial DNA for PCR reaction were prepared by boiling a bacterial culture in 200 µl of PreMan® Ultra Sample Preparation Reagent (Applied Biosystem, Foster City, CA) for 10 min, followed by centrifugation. Initially primers were designed to amplify the quinolone resistance-determining region (QRDR) (Yoshida *et al*., 1990) of *gyrA*.

The gyrase A sequences of *E. coli* was obtained from the GenBank (GenBank accession number: X06373) (Swanberg and Wang, 1987). Primers and probes were designed on the conserved region using Vector NTI® 10.1 software (Invitrogen, Carlsbad, CA), such that the mutant *E. coli* strain has two one-bp mutations in the region for the bodipy 630/650 probe as shown in Fig 1. The length of amplification product was 310 bp with the two primers and probes designed specific for *gyrA* corresponding to bases 152 to 427 of the *gyrA* gene that included all point mutations within the QRDR (Fig 1). The carboxyfluorescein (6-FAM) probe, which was the unpurified 3' labeled FRET energy donor, was excited by 488 nm light. The bodipy 630/650 probe, which was HPLC-purified 5'-labeled, and 3'-phosphorylated, was used as an acceptor probe (von Ahsen *et al*., 2000). Fluorescence emitted from bodipy 630/650 probes served for detection of gyr A at 640 nm.

We designed only one single probe based on the sequence of the wild type so that the bodipy 630/650 probe bridged the two potential point mutations in codons 83 and 87 of *gyrA*. This enabled discrimination of the wild-type and mutated isolates based on the MCA.

Quantitative real-time FRET-PCR

Quantitative determination of *E. coli* was performed by real-time FRET-PCR in a LightCycler (Roche Diagnostics), and reaction chemistry was performed as described (Wang *et al*., 2004). Reactions were performed in glass capillaries with 5 µl of sample nucleic acids or standards and 15 µl of 1.33 x master mixture. Each 20 µl reaction contained 2.0 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 0.2 U heat-labile uracil-N-glycosylase (Roche Molecular Biochemicals, Indianapolis, IN).

Thermal cycling consisted of 18 high-stringency step-down cycles followed by 25 fluorescence acquisition cycles. Eighteen step-down thermal cycles included the following steps: 6 cycles at 95°C for 15 s, 72°C for 30 s; 9 cycles at 95°C for 15 s, 70°C for 30 s; 3 cycles at 95°C for 15 s, 68°C for 30 s, 72°C for 30 s. The fluorescence acquisition cycling consisted of following steps: denaturation at 95°C 15 s, annealing at 56°C for 8 s, 66°C for 30 s, and extension at 72°C 30 s. PCR products were purified for automated DNA sequencing with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Nucleotide sequence determination was performed using an ABI automatic DNA sequencer (Model 377; Perkin-Elmer) at the Genomic Sequencing Laboratory, (Auburn University, Auburn, AL, USA) by using the described forward and antisense primers in Figure 1. DNA sequences were analyzed by using Vector NTI® 10.1 software (Invitrogen, Carlsbad, CA).

Quantitative standards were produced by PCR amplification with dTTP, 4 % MetaPhor agarose gel-purification, and quantification of the fragment by Pico-Green assay (Invitrogen, Carlsbad, CA). Standards were verified by DNA sequencing, and used at 10^4, 10^3, 10^2, 10, and 0 copies per 5 µl in $T_{10}E_{0.1}$.

The melting curve for the annealing of the PCR product with the FRET probes was determined by monitoring the fluorescence of channel F2/F1 from 55 to 80°C with a temperature transition rate of 0.1°C per second. Specificity of the PCRs was confirmed by a lack of amplification of extracted DNAs from *Salmonella*, *Bartonella*, and *Yesinia*. The size of amplification products was verified by electrophoresis of 1 µl of the PCR product on 2% agarose gels.

PCR discrimination of fluroquinolone resistance in canine urine specimens

We examined the sensitivity of our PCR assay in the detection of *E. coli* in urine samples in 3 resistant isolates selected among the 70 clinical samples known to have mutations in codon 83 and 87. In this experiment, we performed the assay on dilutions of original urine samples such that the bacterial counts/ml for each sample was, in colony forming units (CFU): 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 0 CFU/ml. Furthermore, we performed the experiment in two clinical urine samples that had the original bacterial counts of $>10^5$ CFU/ml and were fluoroquinolone-susceptible. For real-time PCR analysis of the *gyrA* gene, DNA was extracted directly from urine samples without previous culturing and purification. The DNA from urine specimens (140 µl) was extracted by using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The extracted DNA (5 µl) was used for real-time PCR assay. The FRET PCR amplification was performed as described above.

Results

Genetic analysis of the *gyrA* QRDR

The DNA sequences of the *gyrA* gene fragments for the wild type and *gyrA* mutatnt clinical isolates; including the QRDR (nucleotides 199 to 319 of the corresponding *E. coli* sequence) and excluding the primer sequences are illustrated in Figure 1. The *gyrA* QRDR sequences of the clinical *E. coli* isolates (IDM 15, AU 26, MS 1, MS 10) were aligned and compared to the *E. coli* type strain (ATCC 11775; GenBank accession number: AF052254) reported by Weigel *et al*. (1998) and with the *E. coli* K12 *gyrA* sequence reported by Swanberg and Wang (1987) (GenBank accession number:

X06373). Four nucleotide differences were identified at the following positions: 255, C to T; 267, T to C; 273, and C to T; and 300, T to C; all of these substitutions were nonsense. Mutations were found in isolates MS 1 and MS 10 at the following positions: 248, C to T; 259, G to A of the corresponding codons 83 and 87, respectively (Fig 1).

The amino acid sequences of the QRDRs for the six *E. coli* isolates were highly conserved. The QRDR amino acid sequences of *E. coli* (ATCC 11775, IDM 15, AU 26) were identical to the *E. coli* QRDR (*E. coli* K12). Sequence analysis of *E. coli* (MS 1 and MS 10) QRDRs revealed that two conserved substitutions were detected: Ser-83 to Leu-83, and Asp-87 to Asn-87. Furthermore these amino acid substitutions were associated with a high level of resistance to enrofloxacin (MIC >32 μg/ml).

Real-time FRET-PCR for detection of mutations in *gyrA* sequences

In this experiment we were able to differentiate *E. coli gyrA* mutants (ENR^r) from wild-type susceptible isolates. The quantitative interpretation and the melting curve analysis are illustrated in Fig 2. These results were compared with susceptibility profile results for those isolates. PCR amplification progressed efficiently with different starting DNA concentrations. This can be observed by parallel slopes of the linear phase of the amplification curve corresponding to different starting template numbers (Fig. 2A). Our initial results indicated that the amplicons of the resistant-type *gyrA* isolates formed a less-stable hybrid with bodipy 630/650 probe than did the susceptible ones. The ENR^r isolates MS-1 and MS-10 exhibited T_m of 61°C, as compared to the susceptible isolates IDM-15 and AU-15 which exhibited a T_m of 71°C (Fig. 2B). In this experiment the ΔT_m (defined as the T_m of the wild type − the T_m of the mutant) was 10°C. Agarose gel

electrophoresis of the amplified products confirmed the presence of the 310-bp amplicon for the four positive clinical isolates. Furthermore, the presence of *E. coli gyrA* in these four clinical samples was also confirmed by sequencing the PCR products and comparing these to published wild-type sequences (Fig 2C). The results of MCA were consistent with the sequencing results which revealed the presence of two transitions C/T and G/A mismatch of the corresponding codons 83 and 87, respectively. These results are in agreement with those obtained with the FRET-PCR assay which can directly detect the single nucleotide polymorphisms (SNP) in *E. coli gyrA* from the clinical samples.

Robustness of the assay in discrimination of mutations in the *gyrA* gene in 66 clinical *E. coli* samples

After the DNA and amino acid sequences of the QRDRs from the quinolone-susceptible type isolates were determined, the PCR assay was applied to clinical samples of 66 *E. coli* isolates obtained from canine and feline clinical specimens. Clinical *E. coli* isolates were selected to represent a wide range of ENR MICs (Table 1). An excellent agreement was obtained between the MCA, the MIC results, and gene sequencing performed on the majority of the isolates (Fig 2 and 3). Most of the resistant isolates had T_m that were easily differentiated from those generated with ENR susceptible isolates (Fig 2B). Based on different melting temperatures, the results could be categorized into 3 major groups. In group I the T_m was 61°C, while in group III the T_m was consistently 71°C (Fig 2B). Those isolates in group I expressed double mutations at codons 83 and 87 and exhibited high MIC range (8- >128 μg/ml) (Fig 3). In contrast, the isolates in group III had no mutation, and were associated with low MIC range (0.06- 0.5 μg/ml). The

majority of the isolates were grouped into group I or III. Interestingly, a new emerging group II, which consisted of 9 isolates, had an intermediate T_m that it was easily differentiated from those generated with resistant or susceptible-type templates of group I and group III, respectively. In group II, T_m decreased and formed less stable hybrid compared to group III. We performed multi-sequence alignment analysis on QRDR of *gyrA* and included the codons alterations in ENR^r resistant and -susceptible clinical isolates (Fig 2C). Six isolates in group II exhibited intermediate T_m of 68°C (Fig 3). This result had a match with the single mutation that involved C to T transition in codon 83 of *gyrA* (Fig 2C) and was associated with decreased susceptibility or low levels of resistance to ENR^r (MIC range= 0.25-4 μg/ml; ENR_{BP} = 4 μg /ml [CLSI, 2008]) (Fig 3). This substitution changed the amino acid sequence from Ser-83 to Leu-83. One isolate has been identified to have T_m of 69°C (plotted as T_m of 68°C in Fig 2B). This isolate approached the break point of susceptibility to enrofloxacin (MIC = 2 μg/ml) (Fig 3) (CLSI, 2008) and a substitution that changed the amino acid sequence from Asp-87 to 87-to-Gly (Fig 2C). Furthermore, 2 isolates displayed T_m of 66.5°C (Fig 2B, 3). For those isolates, multi sequence analysis revealed one nucleotide mismatch at codon 85 that involved T to C (Fig 2C). All of these substitutions were nonsense. This corresponded well with the susceptibility results (MIC = 0.06 μg/ml) for those isolates (Fig 3).

The real-time PCR assay amplified products in 70 of 70 samples (100%). The assay precisely detected intermediate or high level resistance among 70 different clinical *E. coli* isolates in 42 of 48 for a sensitivity (correctly predicting true positives, that is either intermediate or high level of resistance) of 87.5% (95% confidence interval [CI] = 75% to 95.3%) corresponding resistant cultures and correctly identified 22 of 22 for a

specificity (correctly identifying true negatives or susceptibe) of 100% (95% CI = 87.3% to 100%) susceptible clinical cultures. The assay discriminated between clinical levels of resistance (i.e. intermediate or low level of resistance, reflecting a single mutation, versus high level of fluoroquinolone resistance, reflecting double mutations in *gyrA*) (Fig 3). The specificity of the PCR primers for *E. coli* was tested using other bacterial species such as *Salmonella*, *Bartonella*, and *Yesinia*, and the FRET signals were not observed in those species. These results confirm the excellent specificity of the primers, discriminatory power of the hybridization probe assay and the utility of this assay to be used clinically.

Sensitivity of PCR amplification in detection of flouroquinlone resistance in 5 clinical urine samples

After extraction of DNA, PCR amplifications were performed successfully in randomly selected 5 (susceptible, n=2; mutants, n=3) clinical urine specimens. MCA revealed that the *Tm* in the wild and mutant type of *E. coli* found in the urine samples produced a similar pattern of results as those in the 70 clinical samples: 61°C and 71°C *Tm* for the wild and the double mutant isolates, respectively. PCR successfully detected bacterial dilution counts ranging from as high as 10^6 CFU/ml to as few as 10 CFU/ml, the latter corresponding to the template DNA concentrations of 19 genome copies.

Discussion

E. coli is emerging as a pathogen that causes a variety of diseases in veterinary medicine (Hagman and Kühn, 2002; Chen *et al*., 2003). The trend of increasing resistance to fluoroquinolones has been described by many investigators (Cooke *et al*.,

2002; Cohn *et al*., 2003), and its association with resistance to other antimicrobial agents (Cooke *et al*., 2002; Karlowsky *et al*., 2003, 2006) has stimulated the need for a rapid method for the detection of fluoroquinolones. In general, the mutations in DNA *gyr A* confer resistance to fluoroquinolone in both gram-negative and gram-positive organisms (Hooper, 1999). In *E. coli* the mutations in *gyrA* leading to fluoroquinolone resistance have been well characterized (Yoshida *et al*., 1990; Heisig *et al*., 1993; Conrad *et al*., 1996); thus a single point mutation in *gyrA* results in increased resistance to fluoroquinolones (Willmott and Maxwell, 1993), and a higher-level of resistance is associated with double mutations in codon 83 and 87 of *gyrA* (Heisig *et al*., 1993; Vila *et al*., 1994; Conrad *et al*., 1996). The substitution in the amino acid Ser-83 was considered to be the first step, for an organism, of becoming resistant to fluoroquinolones (Heisig and Tschorny, 1994). The contribution of mutations in the ParC subunit of topoisomerase IV and decreased intracellular drug accumulation play a secondary role and cause an increase in the level of resistance (Oram and Fisher, 1991; Livermore *et al*., 2002). Accordingly, we have developed a specific and rapid PCR assay to detect ENR^r isolates of *E. coli* using real-time FRET-PCR. The assay detects mutations within *E. coli gyrA* using a single bi-probe in as low as 4 copies of purified target sequence. We were able to determine if the *E. coli* were ENR^r within 1-2 hours using MCA. Our assay has promising value, exhibiting a high sensitivity in the early determination of antimicrobial susceptibility to fluoroquinolones of non-isolated clinical urine samples (i.e. directly from an infected urine specimens). Although we studied only enrofloxacin as model to study fluoroquinolone resistance, a previous study indicated that this would result in resistance to other second generation fluoroquinolones (Sanchez *et al*., 2002). In a clinical

perspective, this is an important finding because the assay is rapid and specific for *gyrA*, thus reducing time consumption and the possibility of cross contamination that may occur with other methods.

We were able to design *E. coli*-specific PCR primers and FRET probes based on nucleotide sequence alignment of the QRDRs of *gyrA* from a clinical *E. coli* culture collection. The FRET assay identified all the *E. coli* isolates tested, while failing to amplify the chromosomal DNA of other bacterial species. The real-time FRET-PCR assay discriminated between wild-type *E. coli* and other *E. coli* isolates that carry a C to T transition in codon 83 and G to A in codon 87 of *gyrA*. Furthermore, we used touch-down thermal cycling for our amplification process in which several gradual high-stringency annealing temperatures preceded the fluorescence acquisition of the real-time PCR (Marchand *et al*., 2003). This increased the specificity of the PCR amplification.

The classical standard methods for determining fluoroquinolone resistance based on CLSI guideline (CLSI, 2008) are tedious, time consuming, and generally result in a 2 to 5 day lapse between collection and reporting. Other molecular techniques such as mismatch amplification mutation assay (MAMA) combined with DNA sequencing (Qiang *et al*., 2002) have been developed for the detection of ciprofloxacin-resistant in clinical *E. coli* isolates. This method required using the conventional PCR based technique that required post amplification process such as gel electrophoresis and special expensive equipment for sequencing. Furthermore, the cross contamination is another drawback and may occur as a result of reopening the tubes at the end of the PCR amplification cycling (Kaltenboeck and Wang, 2005). In contrast, the real-time FRET-PCR format reported here in can detect SNP in *gyrA* in clinical *E. coli* isolates. A

potential additional advantage of the technique is that it allows quantitation of *E. coli* because of the linear relationship observed between initial DNA template and the PCR threshold cycle in real time. This may allow differentiation of infection (which generally requires > 10^5 CFU/ml [Boothe, 2006]) from non-infection.

Other real-time PCR methods have been described previously to detect emerging pathogens (Ko and Grant, 2006; Abdelbaqi *et al*., 2007; Siedner *et al*., 2007) and to differentiate bacterial species as in Campylobacter (Ménard *et al*., 2005). For example, SYBR Green®, hybridization probes, TaqMan probes, Molecular Beacons and Scorpion probes are common real-time detection formats currently used (Kaltenboeck and Wang, 2005). However, SYBR Green is not recommended for tests in which specificity is important as no probe is used and several primers are needed. Other probes used in Taqman , Molecular Beacons and Scorpion require many probes; with two labels needed, design and cost would be greater compared to the need for biprobe labeled once as with the FRET hybridization probe. Thus, rather than four different probes need by TaqMan to detect point mutations (one for each individual point mutation in *gyrA* plus one for the wild type), only one was needed for our method. Taken together, FRET-PCR technique was chosen and proven to be the sensitive and specific method for detection and differentiation of mutations in *gyrA* in clinical *E. coli* samples.

The initial decision to focus on codons 83 and 87 was based on an initial sequence analysis of the QRDR of *gyrA* of 4 clinical isolates, for which *gyrA* mutations were limited to these sites (Fig. 1). Four nonsense mutations have been identified in those isolates in correspondence to the first *gyrA* sequence reported by Swanberg and Wang (1987). This result is in agreement with a previous report by Weigel *et al*. (1998) in

which *gyrA* QRDR sequence of the *E. coli* type strain (ATCC11775) also revealed four nucleotide differences at the same positions. Two of the mutant clinical isolates in this study had C to T transitions at the second position of codon 83 (nucleotide position 248) in *gyrA* and G to A transitions at the first position of codon 87 (nucleotide position 259) in which the MICs were >32 μg/ml. We found only two amino acid positions in GyrA that changed in association with reduced ENR susceptibility (MIC range= 0.25- >128 μg/ml) Ser-83 to Ile-83, Asp-87 to Asn-87 and Asp-87 to Gly-87 (Fig 2C). Thus the double mutations that involve both codons 83 and 87 were associated with high level of resistance (MIC= ≥8 μg/ml) than those isolates with single mutation (Fig 3). In contrast, a single mutation has been identified in our study that changed Ser-83 to Leu-83 and Asp-87 to Gly-87 which was associated with intermediate to low level of resistance (MIC range = 0.25-4 μg/ml). These data were consistent with previous studies (Heisig *et al*., 1993; Willmott and Maxwell, 1993; Vila *et al*., 1994) where the low-level fluoroquinolone resistance in *E. coli* was associated with identical single mutations in codon 83 of *gyrA* and high-level resistance required double mutations in codons 83 and 87. The predominance of the transitions in codon 83 and 87 of *gyrA* among fluoroquinolone resistant *E. coli* indicates a key role for *gyrA* in conferring high-level enrofloxacin resistance. Generally the mutations at codons Ser83 and Asp87 confer higher levels of quinolone resistance than mutations in other codons (Yoshida *et al*., 1990). The ability to form hydrogen bonds and the negative charge of amino acid at these positions seems to be important for quinolone interactions with the DNA gyrase–DNA complex (Vila *et al*., 1996; Sáenz *et al*., 2003).

The range of ENR resistance in the 43 *E. coli* clinical isolates tested for this study was (MIC 4- >128 μg/ml). Those isolates with similar levels of ENR resistance showed similar melting peaks in MCA (Fig 3). Similarly, 27 of the wild-type *E. coli* clinical isolates had low MIC value range (0.06-0.5 μg/ml). We have also sequenced the *gyrA* QRDRs of 5 exceptional *E. coli* clinical isolates that showed in-agreement between MCA and the MIC results. Interestingly, they were enrofloxacin resistant with MIC range (1- 4 μg/ml) in 2 isolates and (16 - >128 μg/ml) in the other 4 isolates (Fig 3). All 5 possessed a sequence identical to the wild isolates. These results emphasize on the importance of other mechanisms beside mutations that could play critical role in the emergence of fluoroquinolone resistance from clinical isolates. In clinical *E. coli*, the AcrAB-TolC system was reported by many investigators to cause MDR including fluoroquinolone via overexpression (Mazzariol *et al*., 2000). These studies indicated that fluoroquinolone-resistant clinical isolates of *E. coli* expressed high levels of *acrB* and *AcrA* of the AcrAB-TolC system (Everett *et al*., 1996, Mazzariol *et al*., 2000). Furthermore, many studies indicated that the combination of efflux pump overexpression and a mutation(s) in a topoisomerase gene give rise to clinical levels of resistance to fluourquinolone (Mazzariol *et al*., 2000). However, the overexpression of AcrAB-TolC system in *E. coli* may confer high level of resistance to fluoroquinolone regardless of mutations topoisomerase genes. This part can be investigated further in future utilizing the efflux pump inhibitor MC-207,110 (Phe-Arg-ß-naphthylamide) that was previously identified (Lomovskaya *et al*., 2001).

In a very short time (1-2 hours) we were able to detect the presence of *E. coli* in the urine samples and the presence or absence of point mutations in *gyrA* gene from

clinical specimens without culturing. In all nonisolated clinical samples, *E. coli* was detected in all diluted urine specimens and the detection limit of the test was determined to be 19 genome copies per reaction (10 CFU/ml urine). In cultured samples the assay can detect as low as 4 genome copies per reaction. There is consistent association between mutations in the *gyrA* gene and resistance to fluoroquinolone. In general, our assay exhibited 93% (65 of 70 samples) sensitivity to detect the correct susceptibility genotype for fluoroquinolone resistance among in clinical samples. This high proportion for detection sensitivity can justify using this assay for surveillance programs especially when we are dealing with a large number of clinical samples (≥ 1000 sample/year).

Conclusion

To our knowledge we are the first to develop and report a FRET-PCR assay that can specifically predict the flouroquinolone resistance-associated *gyrA* mutations in *E. coli* using MCA. Our assay proved to be sensitive, reproducible, and quantitative for detection of ENR^r from canine and feline clinical *E. coli* isolates and from nonisolated urine specimens. Furthermore, the FRET-PCR assay is rapid and sensitive for detecting mutations in *gyrA* and this is critical when designing dosing regimens in a clinical veterinary setting. We have proven the specificity of this assay by using different canine and feline *E. coli* clinical isolates (including the nonisolated specimens) and unrelated bacterial species. However, a perfect correlation is not predicted between antibiotic susceptibility profiles and mutations in QRDR using the molecular techniques. Therefore, more experiments are needed to identify the contribution of other mechanisms, most importunately the efflux pump, in the emergence of fluoroquinolone resistance among

clinical *E. coli* isolates. Our development of a real-time PCR assay will help define the mechanisms of fluoroquinolone resistance in *E. coli*. This rapid detection method will provide helpful information when formulating the antimicrobial dosing regimen for clinical cases of *E. coli* infection and will help to reduce therapeutic failure and the emergence of fluoroquinolone and multi-drug resistance from clinical isolates. Furthermore, because of its applicability to uncultured the urine samples, this assay can be used successfully in surveillance and the emergence of fluoroquinolone resistance among clinical samples of *E. coli* over the time.

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TABLE 1. *E. coli* strains and isolates used in this studies and their antimicrobial susceptibility to enrofloxacin.

Strain/Isoltes ID	Tissue Source	Strain donor[c] or reference	Origin	Antimicrobial Susceptibility[d]	
				MIC (µg/ml)	Interpretation
E. coli K12[a]		GenBank X06373 (Swanberg and Wang, 1987)			S
ATCC 11775[a]	Urine	ATCC/GenBank AF052254 (Weigel *et al.*, 1998)		≤0.12[e]	S
N7190284	Urine	IDEXX-MA	Canine	0.06	S
M0846371	Urine	IDEXX-NC	Canine	64	R
R5107645	Urine	IDEXX-MA	Feline	0.06	S
L8059588	Urine	IDEXX-CA	Feline	64	R
M0851881	Urine	IDEXX-NC	Feline	32	R
M0854758	Skin	IDEXX-NC	Canine	64	R
M0858621	Urine	IDEXX-NC	Canine	>128	R
M0825317	Urine	IDEXX-NC	Canine	0.06	S
M0821846	Urine	IDEXX-NC	Canine	0.06	S
M0824507	Ear	IDEXX-NC	Canine	>128	R
I2132223	Urine	IDEXX-IL	Canine	16	R
B5299277	Urine	IDEXX-CA	Feline	4	R
L8070100	Urine	IDEXX-CA	Canine	32	R
L8063713	Urine	IDEXX-CA	Canine	>128	R
L8055239	Urine	IDEXX-CA	Feline	32	R
F5093961	Urine	IDEXX-CA	Feline	2	I
K5262419	Urine	IDEXX-CA	Canine	0.06	S
W6316944	Trachea	IDEXX-IL	Canine	>128	R
D1056271	Wound	IDEXX-IL	Canine	32	R
W6319937	Nose	IDEXX-IL	Feline	>128	R
I3211251	Urine	IDEXX-IL	Canine	0.06	S
C6394833	Urine	IDEXX-IL	Canine	0.06	S
I2473760	Urine	IDEXX-IL	Canine	>128	R
I3195059	Urine	IDEXX-IL	Feline	128	R
M0846371	Urine	IDEXX-NC	Canine	64	R

MS-1[b]	Wound	MS	Canine	>32[f]	R
MS-10[b]	Ear	MS	Canine	>32[f]	R
AU-26[b]	Urine	AU	Canine	0.064[f]	S
IDM-15[b]	Urine	IDEXX-MA	Canine	0.064[f]	S
W7528341	Skin	IDEXX-OH	Canine	64	R
W7534250	Urine	IDEXX-OH	Canine	32	R
D8632999	Urine	IDEXX-OH	Canine	8	R
D1083401	Urine	IDEXX-OH	Canine	64	R
W7538455	Urine	IDEXX-OH	Canine	16	R
L9254925	Urine	IDEXX-CA	Canine	128	R
L9245953	Urine	IDEXX-CA	Canine	2	I
K5693300	Urine	IDEXX-CA	Canine	1	I
I2850490	Fracture	IDEXX-IL	Canine	64	R
I2846835	Urine	IDEXX-IL	Canine	1	I
I2888998	Nose	IDEXX-IL	Feline	64	R
M1213579	Urine	IDEXX-NC	Canine	4	R
L9473547	Urine	IDEXX-CA	Canine	128	R
L9470993	Urine	IDEXX-CA	Canine	>128	R
I4097858	Urine	IDEXX-IL	Canine	64	R
Y5826621	Urine	IDEXX-MA	Canine	128	R
J7261205	Urine	IDEXX-MA	Canine	64	R
W7538320	Vagina	IDEXX-OH	Canine	0.5	S
F5889381	Urine	IDEXX-CA	Feline	0.12	S
L9254729	Urine	IDEXX-CA	Canine	0.12	S
Y5645394	Vagina	IDEXX-MA	Canine	0.25	S
R6559423	Urine	IDEXX-MA	Canine	0.5	S
Y5643881	Pleural fuid	IDEXX-MA	Canine	0.5	S
B5259772	Urine	IDEXX-CA	Canine	0.25	S
L8063901	Urine	IDEXX-CA	Canine	0.5	S
W6319347	Urine	IDEXX-OH	Canine	0.25	S
D1056065	Urine	IDEXX-OH	Canine	0.25	S
I3634766	Urine	IDEXX-IL	Canine	2	I
B6276689	Urine	IDEXX-CA	Feline	0.5	S
D1079284	Urine	IDEXX-OH	Feline	0.5	S
D1079346	Urine	IDEXX-OH	Canine	0.12	S
I3621025	Abdomenal fluid	IDEXX-IL	Canine	64	R
I3620609	Urine	IDEXX-IL	Canine	128	R
B6276670	Urine	IDEXX-CA	Canine	32	R

Q2100506	Abdomenal fluid	IDEXX-MA	Canine	64	R
D1056271	Tracheal wash	IDEXX-OH	Canine	32	R
B6276651	Urine	IDEXX-CA	Canine	64	R
S3590475	Throat	IDEXX-CA	Canine	16	R
R5271946	Urine	IDEXX-MA	Feline	>128	R
D8474601	Urine	IDEXX-OH	Feline	64	R
W7529296	Urine	IDEXX-OH	Canine	64	R

[a] The DNA sequences of these strains were acquired from GenBank.

[b] Isolates were collected between May and September 2005.

[c] ATCC, American Type Culture Collection, Manassass, VA; IDEXX Laboratories, Inc.,Westbrook, Maine; CA, California; NC, North Carolina; OH, Ohio; MA, Massachusetts; IL, Illinois; MS, Mississippi; AU, Auburn University.

[d] The MIC was determined for enrofloxacin according to CLSI guidlines (CLSI, 2008); R, resistant; I, intermediate; S, susceptible.

[e] The MIC for this strain was determined for ciprofloxacin (Weigel *et al*., 1998).

[f] The MIC was detemined using the E-test® (Epsilon; AB Biodisk, Solna, Sweden) according to CLSI guidlines and interpretive standard (CLSI, 2008).

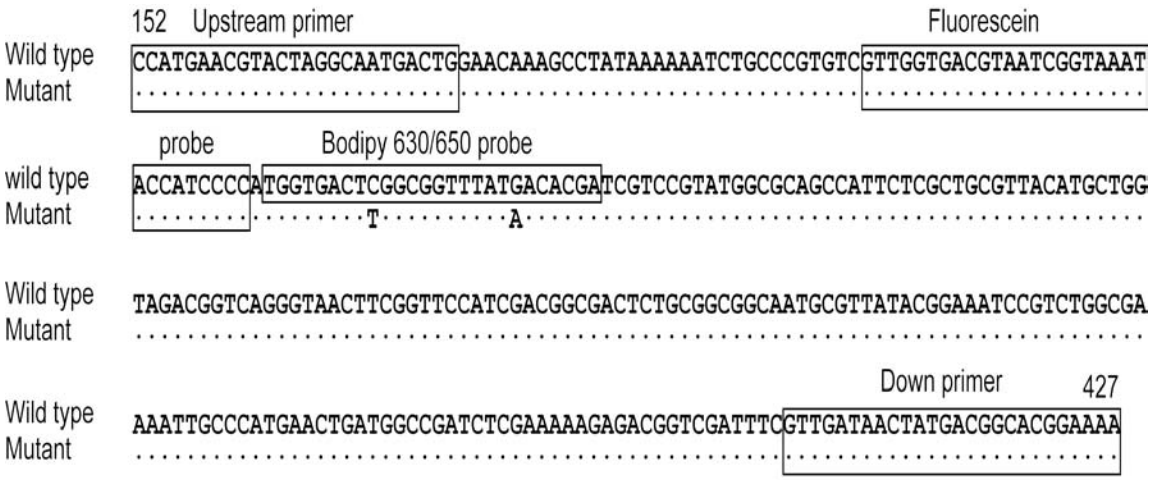

Figure 1. DNA sequence alignments of the *gyrA* QRDRs of *E. coli* isolates including wild isolates and mutant clinical isolates with Ser-83 and Asp-87 mutations.

Consensus indicates nucleotide positions identical to the corresponding *E. coli gyrA* sequence. PCR primers are labeled. Probe 1 (Fluorescein) and probe 2 (Bodipy 630/650) are shown between the upstream and down primer. Probe 1 and probe 2 are identical to with the wild-type *E. coli gyrA* sequence. The positions of recurring mutations in Ser-83 and Asp-87 codons are indicated (boldfaced letters) in the mutant-type DNA sequence below probe 2.

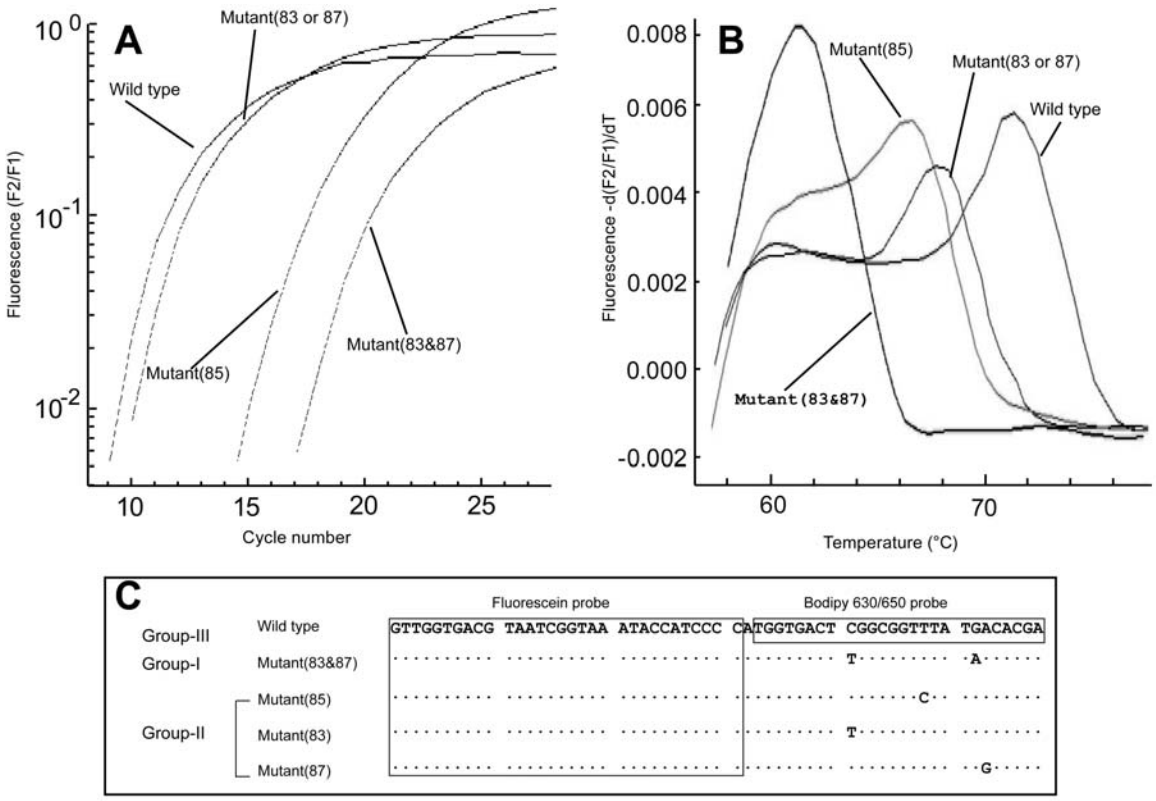

Figure 2. Amplification and melting curve analysis of PCR amplicon containing the QRDR of the *gyr*A gene of clinical *E. coli* isolates.

A, efficient PCR amplification is indicative by parallel slopes of linear phase corresponded to different starting DNA templates. **B**, four different melting peaks represented 3 major groups: I, II, and III were generated from the dissociation of the probes from the wild-type and mutant-type templates. The y axis represents the change in fluorescence as a function of time (dF/dT) while x axis represents the temperature. Different peaks in the MCA corresponded to different mutations are indicated. All mutant isolates produced less T_m than the wild-type template. **C**, multi-sequence alignments of the *gyrA* QRDRs of the clinical *E. coli* isolates and location of the FRET probes used for

real-time PCR amplification. The donor probe (Fluoresceine) and the anchor probe (Bodipy 630/650) are indicated. Nucleotides mismatches are indicated (boldfaced letters) within the anchor probe, and dots indicate nucleotides identical to the corresponding wild *E. coli* sequence.

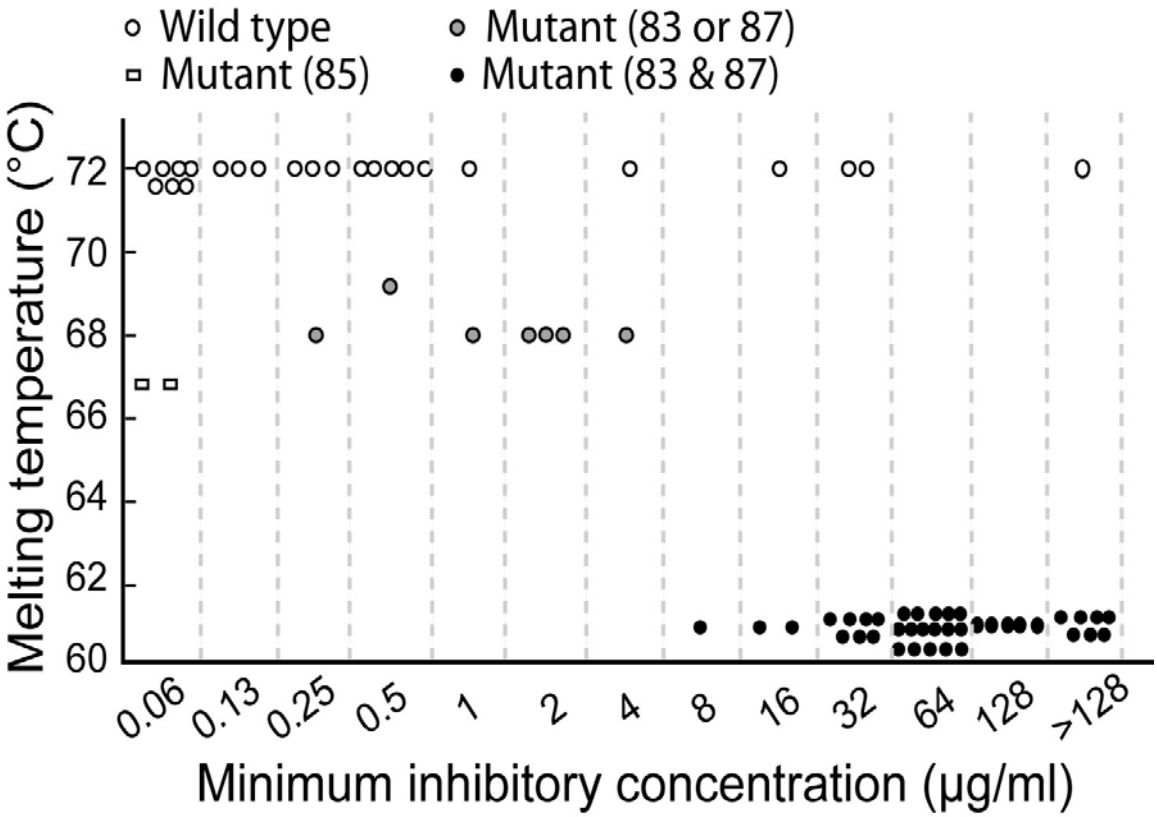

Fig 3. Melting temperatures correlate and predict the enrofloxacin MICs in clinical isolates.

Each dot represents one clinical sample of *E. coli* from dogs and cats. Isolates were carried double mutations in codons 83 and 87 (filled black circle), or single mutation in codon 83 or 87 (filled grey circle), or nonsense mutation in codon 85 (opened triangle), or wild type genotype of *gyrA* (opened circle). Group I includes the mutant (83 and 87) isolates; Group II, includes the mutant (83 or 87) and the mutant (85) isolates; Group III, includes the wild type isolates. Mutant (83 & 87), samples (n=37) with a melting-curve temperature of 61°C corresponding to a mutant genotype in codon 83 and 87 of *gyrA* and had high MICs range (8 - >128 µg/ml); mutant (85), samples (n=2) with a melting-curve temperature of 66.5°C corresponding to a mutant genotype in codon 85 of *gyrA* and had

low MIC values (0.06 μg/ml); mutant (83), samples (n=6) with a melting-curve temperature of 68°C corresponding to a mutant genotype in codons 83 of *gyrA* and had intermediate to low level of resistance (MICs range= 0.06 - 4 μg/ml); mutant (87), samples (n=1) with a melting-curve temperature of 69°C corresponding to a mutant genotype in codon 87 of *gyrA* and had susceptible MIC value (0.5 μg/ml); wild type, samples (n=24) with a melting-curve temperature of 71°C corresponding to a wild-type genotype of *gyrA* and had a wide MICs range (i.e. 0.06 - >128 μg/ml). Interestingly, among the wild type genotype of *gyrA* isolates, 6 out of 24 were exceeding the break point of susceptibility to enrofloxacin (i.e. MIC= 0.5 μg/ml). The MIC results were interpreted according to CLSI guidelines and interpretive standards (CLSI, 2008).

CHAPTER IV

THE CONTRIBUTION OF *GYRA* MUTATION AND EFFLUX PUMPS TO FLUOROQUINOLONE AND MULTI-DRUG RESISTANCE IN CANINE AND FELINE PATHOGENIC *ESCHERICHIA COLI* ISOLATES FROM THE US

Abstract

Objectives: The aim of this study was to investigate the contribution of gyrA and efflux pump and the emergence of fluoroquinlone/MDR resistance among the clinical sample of *E. coli.* Methods: Minimun inhibitory concentrations (MIC) were determined for enrofloxacin and six other drug classes (i.e. beta lactams, aminoglycosides, phenicols, potentiated sulfa, tetracycline, and macrolides) using broth micro-dilution for 536 of *E. coli* clinical isolates with spontaneous infections. Fifty-six of the isolates were further studied for the presence of the broad substrate AcrAB efflux pump system, *gyrA* mutations and their relationship to co-resistance between fluoroquinolone and other drug classes. Phe-Arg-ß-naphthylamide, an efflux pump inhibitor, was used to examine the contribution of efflux pump overexpression.

Results: Fluoroquinolone resistance was significantly associated with a multidrug resistance phenotype compared with fluoroquinolone susceptible isolates. The results suggest that double mutation in *gyrA* can confer high level of resistance to the majority of

fluoroquinolone resistant isolates, and that inhibition by EPI to the efflux pump, presumably AcrAB efflux pump would (i) decrease the intrinsic level of the MICs to FQ in susceptible isolates, (ii) decrease the MICs below the susceptible break point for FQ-R isolates even with single mutation in *gyrA*, (iii) decrease the magnitude of resistance in highly resistance isolates in the presence of double mutations in *gyrA* (IV) decrease the MICs below the susceptible break point for some of the highly FQ-R isolates without *gyrA* mutations, and (V) decrease the resistance to structurally unrelated drugs, thus reducing the incidence of MDR.

Conclusion: Our data provide further evidence that fluoroquinolone use and subsequent resistance can adversely affect the susceptibility of *E. coli* to multiple drug classes.

Introduction

Escherichia coli is a major cause of urinary tract infections and has a significant role in infections of other tissues in dogs and cats (Chen *et al*., 2003; Hagman and Kühn , 2002; Wernicki *et al*., 2002). Fluoroquinolones (FQ) 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 FQ from canine and feline clinical isolates that has emerged in the last decade (Cohn *et al*., 2003; Cooke *et al*., 2002; Guardabassi *et al*., 2004). Because FQs are used both by veterinarians and physicians as first line therapy in USA and Europe (Cohn *et al.,* 2003; Goettsch *et al.,* 2000), 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)

(Guardabassi *et al*., 2004; Lanz *et al*., 2003; Sanchez *et al*., 2002; Talan *et al.,* 2008). Furthermore, FQ resistance to *E. coli* is often associated with multi-drug resistance (MDR) as it has been demonstrated for clinical *E. coli* isolates collected from dogs and cats with spontaneous infections (i.e. non experimentaly induced) (Cooke *et al*., 2002; Karlowsky *et al*., 2006; Sanchez *et al.*, 2002; Shaheen *et al.,* 2008).

The most common mechanism of resistance to quinolones in *E. coli* are mutations in the genes that encode the subunits of the quinolone target topoisomerases, specifically DNA gyrase (topoisomerase II, in *gyrA*), and topoisomerase IV (in *parC*) (Everett *et al*., 1996; Piddock, 1999; Vila *et al*., 1996). These mutations are located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and its homologous region of the *parC* gene (Heisig, 1996; Oram and Fisher, 1991; Piddock, 1999). Mutations in *gyrB* and *parE* genes are less prevelant and rarely contribute to quinolone resistance (Giraud *et al*, 2001; Vila *et al*., 1996). Double mutations in *gyrA* gene are generally required for high-level of resistance (Conrad *et al*., 1996; Heisig *et al*., 1993; Vila *et al*., 1994), whereas mutation(s) in *parC* are less frequent and are associated with lower level resistance (Bagel *et al.,* 1999; Everett *et al.,* 1996; Heisig, 1996). Beside mutations in the QRDR, resistance towards quinolones is now known to be mediated by transfersable resistance genes (i.e. *qnrA, B, C, S*), enzymatic modification (i.e. *aac(6') Ib-cr*) or specific efflux pumps (*qepA*) (Cattoir and Nordmann, 2009; Park et al., 2006; Robicsek et al., 2006a, 2006b; Yamane et al., 2007). Although this mechanism confers low level of resistance alone, their presence was found to enhance the frequency of selection of chromosomal mutatants upon exposure to FQs (Park et al., 2006; Robicsek et al., 2006a, 2006b).

Although a common cause of FQ resistance, mutations in *gyrA* and *par*C should

not directly cause MDR. As such, mechanisms of resistance that involve other classes of antimicrobials must be involved in the emergence of MDR. Among the other mechanisms by which organisms become resistance to FQ is a decreased intracellular, reduced drug concentration by increasing efflux pump activities is a major mechanism for gram negative bacteria (Everett *et al.,* 1996; Giraud *et al.,* 2001; Kern *et al.,* 2000). Among the different efflux transport proteins, the AcrAB-TolC system is most active for *E. coli*. This system belongs to the Resistance Nodulation Division (RND) family of transporters. This system transports a broad range of substrates, including drugs, endogenous substrates and toxins (Piddock, 2006). Among the drug substrates for this system are chloramphenicol, lipophilic ß-lactams, FQs, tetracycline, rifampin, novobiocin, fusidic acid, and nalidixic acid (Piddock, 2006; Sulavik *et al.,* 2001). It is assumed that the evolutionary role of MDR efflux pump is to protect the bacteria against hostile environments (i.e. through the transport of harmful substance out of the bacterial cell). Enteric pathogens (i.g. *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 (MA *et al.,* 1997; Thanassi, *et al.,* 1997). However, over expression of the AcrAB-TolC system in *E. coli* has been demonstrated to be insufficient to cause resistance to ciprofloxacin, chloramphenicol, tetracycline, and cotrimoxazole (Piddock, 2006; Sulavik *et al.,* 2001). It is the combination of a mutation with over expression of an efflux pump that gives rise to high level of resistance to FQs (Mazzariol *et al.,* 2000; Mazzariol *et al.,* 2002). Thus the induction of AcrAB-TolC efflux pump by efflux pump substrate, such as FQs, can cause an increase in the level of resistance to FQs and the emergence of MDR phenotypes (Kriengkauykiat *et al.,* 2005).

The increase of the 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 (EPI) may enhance the activity of some antimicrobials, particularly for gram negative bacteria, including the Enterobacteriaceae (Piddock, 1999). Among the inhibitors of the AcrAB-TolC system, EPI Phe-Arg-ß-naphthylamide reduces resistance caused by the RND efflux systems and can reverse the resistance for antimicrobials that are substrate of AcrAB-TolC efflux pump.

The association of FQ and MDR is generally not addressed in veterinary medicine especially from pet animals (Cohn *et al.,* 2003; Cooke *et al.,* 2002; Lanz *et al.,* 2003; Meunier *et al.,* 2004). Studies addressing these mechanisms have been largely limited to human strains, or isolates of public health concern, such as those collected from food animals (Everett *et al.,* 1996; Giraud *et al.,* 2001). The purpose of this study was to describe the combined role of topoisomerase mutation and efflux pump activity in pathogenic canine and feline *E. coli* collected from animals with disease. Isolates were phenotyped using standard micro-broth dilution susceptibility testing. The level of resistance to FQ was determined based on MIC. In isolates resistant to FQ, target mutation(s) in *gyrA* gene were characterized using fluorescence resonance energy transfer (FRET) PCR. The contribution of efflux pumps to FQ resistance and MDR phenotypes was then tested based on the differences in MIC for each drug in the absence or presence of the EPI.

Materials and methods

Bacterial isolates and culture conditions

Canine and feline pathogenic *E. coli* isolates (n = 536) were acquired from clinical veterinary microbiology laboratories between May and December 2008. All isolates were submitted by veterinary practitioners treating canine or feline patients with infections associated with the culture. The isolates were collected throughout the US by contributing laboratories. These laboratories identified and performed the susceptibility testing on the isolates. At the Clinical Pharmacology Laboratory (CPL) of Auburn University, isolates were plated on CHROMagar™ Orientation (Becton, Dickinson, Franklin Lakes, NJ) for differentiation and confirmation.

The antimicrobial susceptibility testing with and without EPI

Antimicrobial susceptibility testing was performed using custom made micro-dilution susceptibility plates (TREK Diagnostic Systems, Cleveland, OH) according to Clinical and Laboratory Standards Institute (CLSI) guidelines and interpretive standards (CLSI, 2008). Two panels of 13 antimicrobial agents were tested including ampicillin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, meropenem, enrofloxacin, gentamicin, doxycycline, chloramphenicol , and trimethoprim/sulfamethoxazole (Table 1). For standard susceptibility testing, cells were harvested and suspended in 4.5 ml of 0.9% normal saline and adjusted to 0.5 McFarland standard turbidity (~10^8 CFU) using SENSITITER® Nephelometer (TREK Diagnostic Systems, Cleveland, OH). Microdilution plates were incubated at 35°C for 20-24 hours. The results were recorded as true value MIC using the SENSITITER® VIZION system

(TREK Diagnostic Systems, Cleveland, OH). For quality control purposes, *E. coli* ATCC® 25922 (American Tissue Cell Culture, Manassas, VA) and *Enterococcus fecalis* ATCC® 29212 (Manassas, VA) were used. Each isolate was then designated as resistant (R), intermediate (I) and susceptible (S) to enrofloxacin (ENR) and other drugs using CLSI guidelines and interpretive standards (CLSI, 2008).

Multidrug resistance (MDR) was defined as resistance to two or more classes of antimicrobial agents. Class designations and MIC ranges for custom made micro-plates are indicated in Table 1.

In preliminary studies, EPI (Phe-Arg-ß-naphthylamide; Thermofisher, Pittsburgh, PA) was prepared first in 11 ml of Mueller-Hinton broth (MHB), then added in a volume of 0.5 µl into custom made microdilution plates (each well containing 1 µl final volume), such that the final concentrations were 10, 25, 50, 100 and 120 mg/L. Inhibition at 100 mg/L of Phe-Arg-β-naphthylamide did not appear to impact growth conditions; 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 that represent a wide range of enrofloxacin MICs for further studies to characterize the roles of mutation and pump overexpression in FQ resistance. Most (43 of the 56 isolates of *E. coli*) of these clinical isolates were multidrug resistant, and fourteen fully susceptible isolatess were also included in this work. Mutations were determined by FRET PCR assay (As described in Chapter III), and and pump over expression was studied using EPI. Based on MIC and CLSI guidelines (CLSI, 2008), *E. coli* isolates were designated as susceptible (S, n= 14), intermediate (I, n= 5) or resistant (R, n= 37).

An efflux pump-overexpressed phenotype was considered evident for any isolate for which the MIC for ENR decreased at least four fold (two tube dilutions) when the MIC was determined in media containing the EPI compared to media not containing EPI. In addition to ENR, the impact of the EPI on changes in MIC was determined for 13 other drugs.

Detection of *gyrA* gene mutations by FRET-PCR

Bacterial DNA for PCR reaction were extracted using PreMan® Ultra Sample Preparation Reagent (Applied Biosystem, Foster City, CA) following the manufacturer's instructions.

The forward and reverse primers and probes designed specific for gyrA are listed in Table 2. The length of the amplified product was 310 bp.

The copy number of *E. coli* gyrA was determined by real-time FRET PCR in a LightCycler (Roche Diagnostics) (Wang *et al.,* 2004). Each 20 µl reaction contained 2.0 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 0.2 U heat-labile uracil-N-glycosylase (Roche Molecular Biochemicals, Indianapolis, IN), 15 µl of 1.33 X master mix, and 5 µl of sample nucleic acids. The primers and probes were designed using by using Vector NTI® 10.1 software (Invitrogen, Carlsbad, CA) showed in Table 2. The execution protocol for stringent PCR and melting curve analysis followed the previous description (Described in details in Chapter III; Lin *et al.,* 2008).

Statistical analysis

MIC_{50} and MIC_{90} were calculated for each drug class and for all 56 isolates subjected to EPI. Furthermore, a cumulative percent of susceptibility was used to compare the MIC of isolates before and after EPI. The odds ratio of resistance in FQ-R versus FQ-S isolates (i.e. resistant to the antimicrobials but FQs) associated with each of the drug classes was determined. The association of resistance of each drug class to resistance with ENR was investigated. The proportions of resistance to each drug class in the ENR-S versus ENR-R was calculated using the Fisher Exact Test ($P < 0.05$ were considered to be statistically significant). Analyses were performed using the statistical software MINITAB® 15 package (Minitab Inc., PA, USA). The cumulative percent of susceptibility was calculated using SWIN® Epidemiology Module (TREK Diagnostic Systems, Cleveland, OH).

Results

Fluoroquinolone susceptibility patterns

Seventy one percent (381/536) of the isolates expressed resistance to one or more drugs. Of the 381 isolates expressing a resistant phenotype, 20% (n=76) included FQ-R resistance. The FQ-R was significantly associated with MDR, and 98.7% of FQ-R expressed MDR phenotype compared to only 32% of the FQ-S (OR 158.4, CI 27.3–915.4) (Table 3). Among all antimicrobial drug classes, FQ-R was most frequently associated to resistance to beta lactams (95%). Comparison between FQ-R and FQ-S isolates revealed that FQ-R isolates were significantly associated with doxyxycline resistance (68%; OR 19.1, CI 10.6-35.3), gentamicin resistance (42%; OR 9.8, CI 5.2-

18.5), chloramphenicol resistance (39%; OR 2.4, CI 1.4-4.0), and trimethoprim/ sulfamethoxazole resistance (34%; OR 5.8, CI 3.1-11) (Figure 2).

Characterization of fluoroquinolone susceptibilities of canine and feline *E. coli* isolates

A total of 56 (10% of the total) clinical *E. coli* isolates were randomly elected for further characterization of resistance. Isolates represented different MIC and thus resistance levels (susceptible, intermediate or high) for ENR, as well as resistance to other drug classes. Levels of resistance were based on CLSI MIC breakpoints (CLSI, 2008): susceptible at 0.12–0.5 mg/L (n = 14), intermediate at 1–2 mg/L (n=5) or resistant at more than mg/L (n=37) (Table 4).

Target mutations in the *gyrA* gene of the canine and feline isolates of *E. coli*

For susceptible isolates (n=14), the majority (n=12) were free of *gyrA* mutation(s) in the tested region. For intermediate isolates (n=7, MIC= 0.25-2 mg/L), five had a single mutation in *gyrA* (S83L), one had a single mutation in *gyrA* (D87G); while the remaining isolate had no *gyrA* mutation.

All but three resistant isolates (n= 33) expressed double mutations in *gyrA* (i.e. S83L and D87N) (Table 4). The three exceptions carried no mutations in *gyrA* despite the high level of resistance (MIC =16, 32 and 128 mg/L) (Table 4). Similarly, one isolate with ENR resistance of 4 mg/L exhibited no mutation in *gyrA*. This can be attributed to other resistant deteminats such as the mutations in the regulatory genes and their effect on the efflux pump and porins (Piddock, 2006).

Effect of the EPI Phe-Arg-β-naphthylamide

Inhibition of the efflux pump resulted in a reduction in the MIC below the susceptible break point (0.5 mg/L) for isolates with an MIC of 4 mg/L or less, regardless of the presence of a mutation. Additionally, susceptibility was returned for the wild isolates expressing high level resistance by the addition of EPI. For those isolates with two mutations, EPI resulted in a median decrease in MIC of 16 fold (Figure 2). Interestingly, the median decrease in MIC for single mutations was ≥32 fold. It is important to note, however, that although the fold decrease in MIC was still high for isolates with double mutations, the median MICs of ENR for those isolates was still above the resistant (≥ 4 mg/L) breakpoint for ENR after the treatment with EPI. This is in contrast to the EPI-induced decrease in MIC for low or intermediate isolates with single mutation in *gyrA*. For these isolates, the median MIC decreased to ≤ 0.06 mg/L, which is below the susceptible breakpoint. However, in terms of magnitude of decrease in MIC, EPI had the greatest impact on ENR MICs (median decrease ≥267 fold) for the isolates with no mutations (n=5; MIC=1->128 mg/L), compared with those with mutation(s) (Figure 2).

The treatment with EPI also influenced the susceptibility to other selected antimicrobials, including azithromycin, doxycyline, and chloramphenicol. Based on changes in the cumulative susceptibility of azithromycin, the percent of suscebtible isolates below the susceptible and resistant breakpoints was 0% and 46% prior to treatment with EPI compared to 68% and 96% with active efflux pumps (Figure 3a). For doxycyline, the percent of suscebtible isolates below the susceptible and resistant breakpoints was 32% and 57%, without EPI, compared to 98% and 98%, with EPI

(Figure 3b). For chloramphenicol (susceptible and resistant break point of ≤8 mg/L and <32 mg/L, respectively), 57% and 71%, of the isolates were susceptible without EPI, compared to 84% and 93%, with EPI (Figure 3c). The presence of EPI had no impact on the susceptibility of the isolates to gentamicin (Figure 3d).

Discussion

The resistance to FQs has increased since their approval in the late 1980's, with the resistance increasing particularly in the last decade for pathogenic *E. coli* and other gram negative bacteria (Boothe *et al.,* 2006; Cohn *et al.,* 2003; Cooke *et al.,* 2002; Guardabassi *et al.,* 2004). 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 (Shaheen *et al.,* 2008). The purpose of this study was to define the underlying causes of *E. coli* resistance, including MDR and its association with FQ resistance. Specifically, we were interested in the individual or combined effect of target gene modifications and efflux mechanism on the emergence of the FQ/MDR phenotypes of *E. coli* from dogs and cats.

Data from our previous study (Cooke *et al.,* 2002) indicated an increased prevalence of ENR resistance among uropathogenic *E. coli* collected from dogs during 1994 to 1998. These isolates had been collected from patients at veterinary teaching hospitals and private practices throughout the United States. Cohn *et al*. (2003) also documented an increase of FQ resistance in bacteria from canine urinary tract infection between 1992 and 2001 in Missouri. A significant increase in the overall proportion of resistant bacteria (including *E. coli*) to either ciprofloxacin or ENR was demonstrated

separately. These authors concluded that the overall efficacy of FQ remained high (> 80% susceptible), but the data suggested an increase in resistant bacteria isolated from the urinary tract of dogs. Furthermore, Boothe *et al.* (2006) demonstrated that even those isoalates designated as susceptible based on CLSI guidelines were characterized by MIC sufficiently high that only the highest recommended dose would acehive targeted pharmacodynamic indices, that is Cmax/MIC of 10 or more, or AUC/MIC of 125 or more. Similarly, our results indicated a similar pattern of FQ resistance among *E. coli* isolates collected between May and December 2008 in the US. These findings may be expected following the extensive use of these drugs since their approval; FQs were often the first drugs of choice used empirically (without the benefit of culture and susceptibility testing) to treat many infections in dogs and cats (Guardabassi *et al.,* 2004; Lanz *et al.,* 2003; Sanchez *et al.,* 2002).

Several surveillance studies have tracked changes in *E. coli* susceptibilities to FQ and other drug classes over time in humans, but few studies have evaluated the mechanisms that account for this increase in resistance in companion animals (Cohn *et al.,* 2003; Cooke *et al.,* 2002; Lanz *et al.,* 2003; Meunier *et al.,* 2004). Our study suggestes the mechanisms by which this resistance is emerging. First, we investigated the target gene modification as one of the common mechanism that confers resistance to FQ among selected resistant and susceptible clinical *E. coli* isolates. The mutations in DNA gyrase and topoisomerase IV are the most important mechanisms that confer resistance to quinolone (Everett *et al.,* 1996; Piddock, 1999; Vila *et al.,* 1996). In our study, the majority of FQ-R isolates were found to carry double mutations in *gyrA*, whereas intermediate to low level of resistance were found to be associated with single mutation;

a finding consistent with previous reports (Heisig *et al.,* 1993; Vila *et al.,* 1994; Willmott *et al.,* 1993). It is apparent that mutations at codons Ser83 or Asp87 confer higher levels of quinolone resistance than mutations in other codons (Yoshida *et al.,* 1990). The differences in levels of resistance may be attributed to the ability to form hydrogen bonds and the negative charge of amino acid at these positions, which seems to be important for quinolone interactions with the DNA gyrase–DNA complex (Sáenz *et al.,* 2003; Vila *et al.,* 1996). Surprisingly, approximately 9% of the isolates exhibited no mutation(s) in *gyrA*; yet, one isolate expressed intermediate and four expressed high levels of FQ resistance. These results provide a second evidence of the importance of the efflux system and the emergence of FQ resistance/MDR phenotype among clinical isolates of *E. coli*.

It is noteworthy that our study demonstrated, as has others (Cooke *et al.,* 2002; Karlowsky *et al.,* 2006) that many FQ-R isolates exhibit resistance to structurally unrelated antimicrobial agents. Our MDR phenotypes were more likely to be associated with FQ-R (158 times) compared with FQ-S, and that MDR phenotype was expressed to different drug classes. This strong association between FQ-R and MDR suggests that the over expression of efflux pumps contribute to the emergence of MDR phenotype in FQ-R isolates. This might be expected if mutations induce overexpression of the pump and thus confer resistance to a wide range of different drug classes (Piddock, 2006; Sulavik et al., 2001). Therefore, selection for isolates expressing MDR is expected to be induced with previous FQs treatmentbecause these agents are one of the many efflux pump substrates (Piddock, 2006; Sulavik et al., 2001).

We screened for the efficacy of EPI (i.e. Phe-Arg-ß-naphthylamide) to decrease

the magnitude of MICs toward ENR and other drugs based on its ability to potentiate levofloxacin activity (Lomovskaya *et al.,* 2001; Piddock *et al.,* 2006; Renau *et al.,* 1999). Indeed, EPI (i.e Phe-Arg-bnaphthylamide) has been used as to determine the prevalence of efflux pump overexpression phentoypes in *P. aeruginosa* (Kriengkauykiat *et al.,* 2005). We found that the EPI at low concentration (i.e. 25 mg/L) did not effectively decrease MICs to ENR in *E. coli* (data no shown), a finding consistent with previous reports (Kern *et al.,* 2006; Mazzariol *et al.,* 2000; Sáenz *et al.,* 2004). For ENR, at lease 4-fold reduction in the MICs was observed in almost all of isolates, with exception of one isolates that had a MIC= 0.12 mg/L. These results highlight the important of intrinsic resistance of gram negative bacteria to many antimicrobial drugs. This ressitance has been attributed to a constitutive expression of the efflux pump alone, or together with decreased porins expression that could confer a basal level of resistance for those isolates (Everett *et al.,* 1996; Giraud *et al.,* 2001; Kern *et al.,* 2000; Piddock, 2006).

The efficacy of EPI to reduce the MICs was affected by the target mutations in *gyrA*. This effect was evident among isolates carrying one single mutation in *gyrA*, in which the MIC of ENR decreased from intermediate level of resistant to susceptible levels. In contrast, EPI was found to decrease the MICs of ENR in 33 out 35 strains that carried double mutation in *gyrA*. Interestingly, EPI failed to restore the susceptibility to ENR MICs in the presence of double substitutions in the target gene. The presence of efflux pump over expression can contribute to further increase the level of resistance ($\geq$ 8 fold) in the presence of double mutation in *gyrA*. This result is consistent with reports on the importance of combined mechanisms that confer high level of resistance among clinical samples (Fernandes *et al.,* 2003; Mazzariol *et al.,* 2000; Mazzariol *et al.,* 2002).

These data suggest that double mutations provide the mechanism for clinical isolates to become resistant to FQ and have an enhance efflux system that act additively.

Interestingly, EPI decreased the MIC most (≥ 67 fold) for FQ-R isolates carrying no mutations in *gyrA*; the levels of resistance of ENR were restored to wild-type levels. Therefore, it appears that over expression of the efflux pump can have an impact on intracellular drug concentrations, dramatically decreasing the concentration such that over expression of the pump alone is sufficient to cause of FQ-R 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, hyper-susceptible to FQs and other drugs (Oethinger et al., 2000). Similar findings have been demonstrated with other organisms such as *P. aeruginosa*, with deletion of MexAB-OprM efflux pump (Lomovskaya et al., 1999), *Salmonella enterica* serovar Typhimurium DT204, with inactivation of *acrB* (Baucheron *et al.,* 2002) and in *Campylobacter jejuni* with *cmeB* deletion (Luo *et al.,* 2003).

We also demonstrated that EPI reduced the MICs of several antimicrobial drugs. EPI appears to have the strongest inhibitor effect on macrolides (i.e. azithormycin), drugs not normally considered effective toward gram negative organisms. The pump also markedly impacted doxycyline and chloromphenicol. Remarkably, MIC_{90}s for the isolates resistant to azithromycin decreased by > 64-fold in the presence of an EPI compared to 32 for doxycyline resistant isolates and 16 fold for chloromphenicol resistant isolates (Figure 3). The EPI appears to have not impact on the MIC of aminoglycoside (i.e. gentamicin) when tested over the wide MIC range. This is not surprising, as the AcrAB-TolC efflux system in *E. coli* has no effect on aminoglycosides (Piddock, 2006)

compared to that other efflux system (i.e. AcrD), which does impact efflux of aminoglycosides (Nishino and Yamaguchi, 2001; Rosenberg *et al.,* 2000). Furthermore, the EPI did not decrease the MICs of any beta lactams studied here. It may be important to note that the Phe-Arg-β-naphthylamide inhibits efflux of some but not all the substrates of RND pumps, including AcrAB-TolC, because the inhibitor binding site is specific (Lomovskaya and Watkins, 2001).

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* has proven to be a major mechanism that contributes significantly to emergence of high level of resistance to FQ. A single target-based mutation provides only intermediate level of resistance to ENR. Furthermore, we have provided evidence that isolates resistant to FQ were also resistant to other drug classes simultaneously with enhanced efflux pump over expression. We have demonstrated that inhibition by EPI to the efflux pump, presumably AcrAB efflux pump, at the concentrations used in this study would (i) decrease the intrinsic level of the MICs to FQ in susceptible isolates, (ii) decrease the MICs below the susceptible break point for FQ-R isolates even with single mutation in *gyrA*, (iii) decrease the magnitude of resistance in highly resistance isolates in the presence of double mutations in *gyrA* (IV) decrease the MICs below the susceptible break point for some of the highly FQ-R isolates without *gyrA* mutations, and (V) decrease the resistance to structurally unrelated drugs, thus reducing the incidence of MDR. Thus, the increase in the emergence of FQ-resistant and MDR *E. coli* calls for a rational usage of these drugs by veterinarians.

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Table 1. Antimicrobial drugs used in this study

Drug class	Antimicrobial drugs	MIC_{BP} (S/R)[a]	MIC range[b]
Beta lactams	Ampicillin	≤8, ≥32	0.5–256
Penicillins	Amoxicillin/clavulanic acid	≤8/4, ≥32/16	0.25–512
	Meropenem	≤4, ≥16	0.25–512
	Ticarcillin/ clavulanic acid	≤16/2, ≥128/2	2–2048
Cephalosporins	Cefpodoxime	≤2, ≥8	0.12–128
	Cefotaxime	≤8, ≥64	1–1024
	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	0.5–1024
Macrolides	Azithromycin	≤0.5 , ≥8	0.12–256
Aminoglycosides	Gentamicin	≤4, ≥8	0.12–256
Potentiated sulfa	Trimethoprim/ sulfamethoxazole	≤2, ≥8	0.06–128

MIC, minimum inhibitory concentration; BP, break point; S, susceptible break point; R, resistant break point. [a]The MIC beak points were designated according to CLSI guidelines and interpretive standards (CLSI, 2008).

[b]The MIC ranges for custom-made micodilution plates.

Table 2. Primers and probes used in FRET PCR assay and in the melting curve analysis

Primer or probe	Oligonucleotide sequence (5' – 3')	Position[a]	T*m* (°C)[b]
Upstream primer	CCATGAACGTACTAGGCAATGACTG	152-177	57
Down primer	TTTTCCGTGCCGTCATAGTTATCAA	256-427	58
Fluorescein	GTTGGTGACGTAATCGGTAAATACCA TCCCC-(6-FAM)[c]	208-238	66
Bodipy 630/650	(Bodipy-630/650)-TGGTGACTCGGCGGTTTATGACACGA -(Phosphate)	240-265	66.8

[a]The positions of the oliginucleotides correspond to those in GenBank accession number X06373.

[b]T*m*, Theoretical melting temperature.

[c]6-FAM, 6-Carboxyfluorescein.

Table 3. Antimicrobial resistance pattern of fluoroquinlone resistant and susceptible isolates

Isolates with resistance to[a]	**R Cases (FQ-R); *n*=76 (%)**	**Controls (Non FQ-R); *n*=305 (%)**
One class only	1 (1.3)	207 (68)
Two classes	13 (17)	63 (20.6)
Three classes	12 (15.8)	27 (8.8)
Four classes	25 (33)	5 (1.6)
Five classes	18 (23.7)	3 (1)
Six classes	7 (9.2)	0 (0)
MDR phenotype	75 (98.7)	98 (32)

FQ-R, fluoroquinolone-resistant isolates; FQ-S, isolates resistant to all but FQ; MDR, multidrug resistance (resistance to two or more classes of antimicrobial agents).

[a]Each of the following represents a 'unique' class for the purpose of study analysis: enrofloxacin/ciprofloxacin, ampicillin/amoxicillin-clavulanic acid/cephalothin/cepfoxitin/cefpodoxime/cefotaxime/ceftazidime/meropenem/tricarcillin-clavulanic acid, doxycycline, chloramphenicol, gentamicin, Trimethoprim/sulfamethoxazole.

Table 4. Characteristics of *Escherichia coli* isolated from dogs and cats used in this study

Species	Source	QRDR of *gyrA*	Phenotypes of Isolates	Enrofloxacin MIC[a] Without inhibitor	Enrofloxacin MIC[a] With inhibitor	Magnitude of MIC decrease
Canine	Urine	D87G	MDR13	0.5	≤ 0.06	≥8
	Vagina	S83L	MDR26	0.25	≤ 0.06	≥4
	Urine		ENR	1	≤ 0.06	≥16
			ENR, MDR1234	2	≤ 0.06	≥32
			MDR12346	4	≤ 0.06	≥67
		S83L, D87N	MDR12456	8	1	8
			MDR1245	16	2	8
			MDR124	16	1	16
			MDR12346	32	4	8
			MDR146	32	8	4
	Tracheal wash		MDR13456	32	4	8
	Urine		MDR1245, MDR12346, MDR13456, MDR12345	64	4	16
			MDR12456	64	8	8
	Abdominal fluid		MDR12456	64	4	16
			MDR2345	64	8	8
	Fracture		MDR14	64	8	8
	Urine		MDR12456	64	4	16
			MDR1246	128	8	16
			MDR124	128	16	8
			MDR123456, MDR124	128	4	32
			MDR123456	>128	8	≥32
			MDR12346, MDR12345, MDR123456	>128	8	≥16
			MDR1234	>128	16	≥8

	Tracheal wash		MDR123456	>128	8	≥16
	Urine	Wild	NR	0.12	≤ 0.06	≥2
			BL	0.12	0.12	0
			BL, MDR123	0.25	≤ 0.06	≥4
			MDR126, MDR12356	0.5	0.12	4
	Pleural fluid		BL	0.5	≤ 0.06	≥8
	Vagina		MDR125	0.5	≤ 0.06	≥8
	Urine		ENR	1	≤ 0.06	≥16
			MDR1234	16	≤ 0.06	≥267
			MDR12345	32	≤ 0.06	≥530
Feline	Urine	S83L	MDR1234	2	≤ 0.06	≥32
		S83L, D87N	MDR12456, MDR1246	32	4	8
			MDR124, MDR1456	64	8	8
			MDR1246	64	4	16
			MDR1234	>128	8	16
			MDR1245	>128	16	≥8
		Wild	NR	0.12	≤ 0.06	≥2
			BL, NR	0.5	≤ 0.06	≥8
			MDR1234	4	≤ 0.06	≥67
	Nasal cavity		MDR123456	>128	≤ 0.06	>2130

QRDR, quinolone resistance-determining region; MIC, minimum inhibitory concentration; MDR, multidrug resistance (resistance to two or more classes of antimicrobial agents: 1, beta lactams; 2, tetracycline; 3, phenicols; 4, fluoroquinolone; 5, potentiated sulfa; 6, aminoglycosides).

[a]The enrofloxacin MIC was determined in the absence and presence of 100 mg of the efflux inhibitor Phe-Arg-ß-naphthylamide per L.

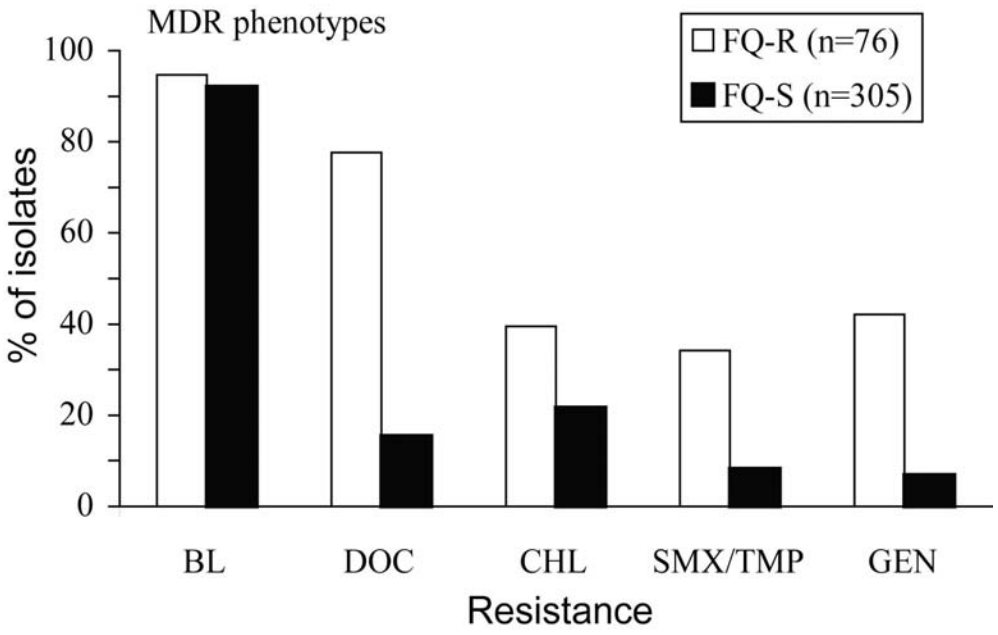

Figure 1. Comparison of the frequency of resistance pattern of FQ-R versus FQ-S isolates to different drug classes. $P < 0.05$ for all comparisons except for beta lactam drugs. FQ-R, fluoroquinolone-resistant isolates; FQ-S, isolates resistant to all but FQ; MDR, multidrug resistance (resistance to two or more classes of antimicrobial agents: BL, beta lactam drugs (i.e. ampicillin/amoxicillin-clavulanic acid/cephalothin/cepfoxitin/cefpodoxime/cefotaxime/ceftazidime/meropenem/ tricarcillin-clavulanic acid; DOC, doxycyline; CHL, chloramphenicol; GEN, gentamicin; SMX/TMP, trimethoprim/sulfamethoxazole.

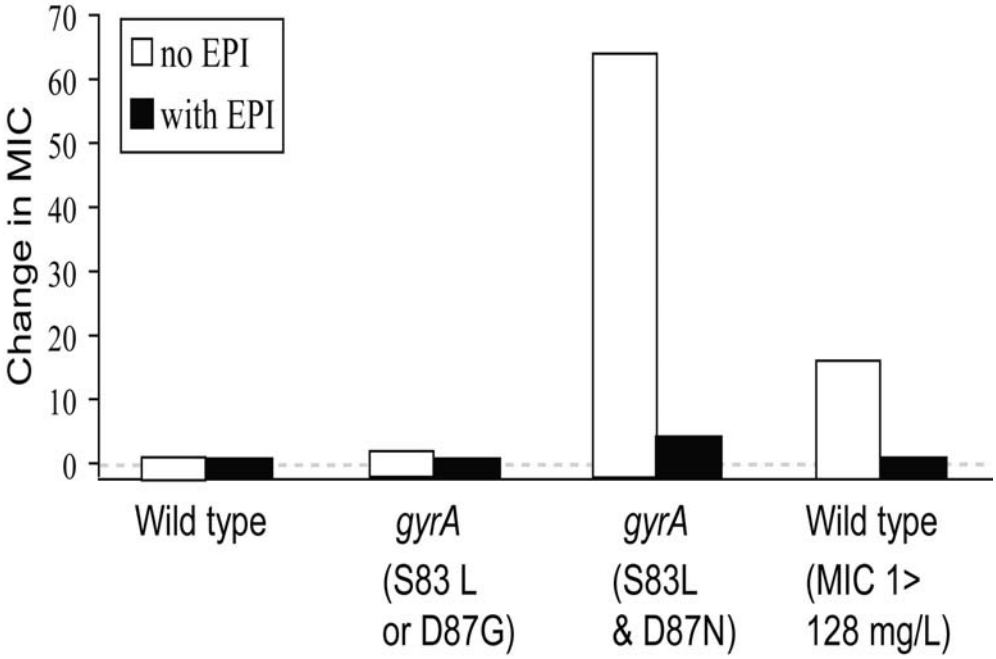

Figure 2. Effect of *gyrA* mutations and EPI on the in vitro MICs of enrofloxacin for 56 clinical *E. coli* isolates isolated from dogs and cats.

With EPI, the median MIC decreased from 0.25 and 2 to ≤ 0.06 mg/L for isolates with wild type and single mutation in *gyrA* (i.e. S83L or D87G), respectively. EPI decreased the median MIC for isolates with double mutation in *gyrA* (i.e. S83 and D87N) by 16 fold, however the MIC is still above the resistant breakpoint (i.e. MIC= 4 mg/L). Interestingly, MIC restored the susceptibility (i.e. ≤ 0.06 mg/L) for isolates (n =4) that had high MICs (i.e. 4- >128 mg/L) and carried no mutations in *gyrA*. EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration.

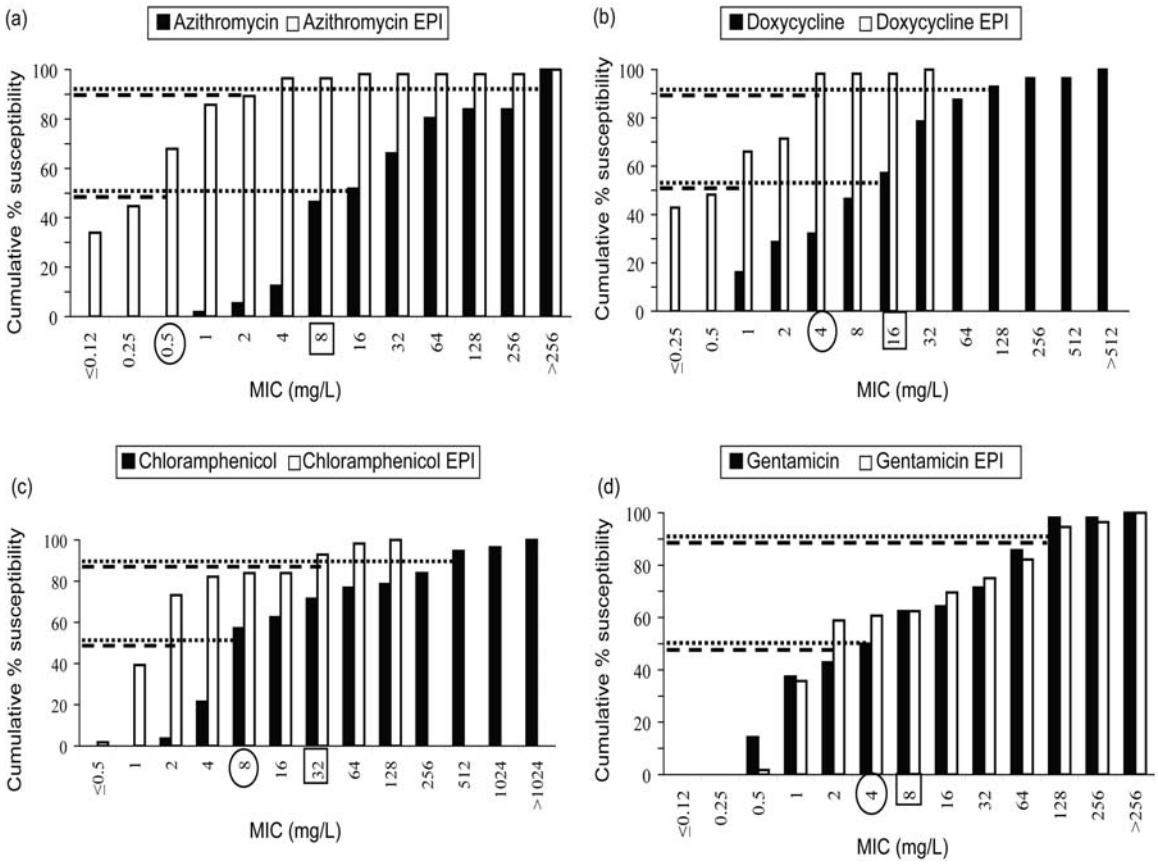

Figure 3. The effect of EPI on the susceptibilities of different antimicrobial agents. Cumulative susceptibility to azithromycin (a), doxycyline (b), chloramphenicol (c), and gentamicin (d) alone or in combination with EPI for clinical *E. coli* isolates from dogs and cats. MIC_{90} and MIC_{50} are represented by thick dashed line (for isolates treated with EPI) or dotted lines (no EPI treatment). MICs in square and triangle represent the susceptible and resistance breakpoints, respectively, and were designated according to CLSI guidelines and interpretive standards (CLSI, 2008). EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration.

CHAPTER V

THE ROLE OF CLASS 1 AND 2 INTEGRONS IN MEDIATING ANTIMICROBIAL RESISTANCE AMONG CANINE AND FELINE CLINICAL *E. COLI* ISOLATES FROM THE US

Abstract

Integrons are mobile genetic elements that incorporate an open reading frame or gene cassette and have an important role in the acquisition and dissemination of antimicrobial resistance genes. Yet their role in the transmission of resistance in bacterial pathogens of pet animals is seldom addressed. The purpose of this study was to describe the incidence of class I and 2 integrons in *Escherichia coli* pathogens ($n = 82$) isolated from dogs and cats with spontaneous infections. The relationship of integrons to antimicrobial resistance was described. Integrons were detected using PCR and then further characterized by restriction fragment length polymorphism (RFLP) analysis and amplicon sequencing. Class 1 was detected in 27% of the total isolates, while only 2.4% ($n = 2$) of the isolates were positive for the presence of class 2 integrons. Integrons negative isolates represent 72% (n= 59) among selected isolates. Eleven gene cassettes were found either alone or in combination with other gene cassettes, which encoded resistance to aminoglycosides (*aadA1, aadA2*, *aadA5, aacA4*, and *aadB*), trimethoprim (*dfrA1*, *dhfrA17*, and *dfrA12*), chloramphenicol (*catB3* and *cmlA6*), and streptothricin

(*sat1*), respectively. The gene cassette *aadA1* (65.2%, n= 15/23) was detected most frequently in class 1 integrons. All integron positive isolates were characterized by resistant to least two drug classes. The role of integrons in mediating resistance was confirmed by curing experiments for three isolates for which resistance was resolved once large plasmids (size range 97-169 kb) carrying the class I integron were lost. To our knowledge, this is the first report of class 2 integrons and antibiotic resistance gene cassettes found in *E. coli* from companion animals in US.

Introduction

E. coli is a common cause of canine and feline urinary tract infection and pyometra (Hagman and Kühn, 2002; Chen *et al*., 2003). Several antimicrobial agents have been used historically to treat these infections in veterinary medicine, including penicillins, cephalosporins, macrolides, lincosamides, fusidic acid, tetracyclines, chloramphenicol, potentiated sulphonamides, aminoglycosides and fluoroquinolones. The use of antimicrobial drugs has been associated with an increase in the trend of antimicrobial resistance among canine and feline clinical *E. coli* isolates in the last decade (Normand *et al.,* 2000b). In addition to the incidence of resistance, the type of resistance expressed by *E. coli* also is changing, with more isolates expressing multi-drug resistance (MDR) in the US and Europe (Normand *et al.*, 2000a; Cohn *et al*., 2003; Guardabassi *et al*., 2004; Shaheen *et al.*, 2008).

The dissemination of MDR phenotypes among *E. coli* isolates may occur as a consequence of horizontal gene transfer mediated by mobile genetic elements, which

include plasmids, transposons, genomic islands, integrons, and natural transformation (Osborn and Böltner, 2002). Integrons play an essential role in facilitating the transit of the resistance genes and contributing to the creation of MDR-phenotypes (Hall and Collis, 1998; Rowe-Magnus and Mazel, 2002). Integrons incorporate gene cassettes by site specific recombination events mediated by integrase genes, which flank the 5' and 3' conserved segments (Collis *et al*., 1993). In addition to the gene (*intI*), the integron structure has a primary recombination site (*attI* site) at the 5'CS and *qacEΔI* and *sulI* at the 3′CS region (Recchia and Hall, 1997; Stokes *et al.*, 1997). Gene (*intI*) encodes for the integrase enzyme that catalyzes the recombination event that involves the primary recombination site (*attI*) as well as a secondary target site (attC site or 59 base element), which is eventually found associated with gene cassette. To date, five classes of integrons (Classes I-V) have been identified based on the character of the integrase gene associated with the cassette. Class 1 integrons are most commonly found among multi-drug resistance in gram positive and negative bacteria (Rowe-Magnus and Mazel, 2002; Fluit and Schmitz, 2004; Mazel, 2006) and are found associated with the Tn21 transposon family (Rowe-Magnus and Mazel, 2002; Mazel, 2006). Additionally, integrons have been also identified and characterized in bacteria from environmental samples (Rosser and Young, 1999; Petersen *et al.*, 2000). More than 80 gene cassettes are associated with class 1 integrons (Mazel, 2006).

Several studies have been conducted in many countries to document and characterize the emergence of class 1 and 2 integrons among *E. coli* clinical samples isolated from dogs and cats (Lanz *et al.*, 2003; Sidjabat et *al.*, 2006; Cocchi *et al.*, 2007;

Kadlec and Schwarz, 2008). Only 22% and 15% of the isolates have been documented to carry class 1 integrons among clinical sample of *E. coli* from Switzerland and Australia, respectively (Sidjabat *et al*., 2006; Cocchi *et al.*, 2007). However, there is a little information regarding the acquisition and dissemination of antimicrobial resistance genes among canine and feline clinical *E. coli* isolates in the US. One study indicated that 30 of 34 nosocomial isolates harbored the *intI1* integrase gene and those isolates were resistant to more than one drug (Sanchez *et al*., 2002).

The dissemination of integrons has been attributed to the extensive use of antimicrobials, particularly in production animals (Cocchi *et al.*, 2007). The judicious use of antimicrobials in dogs and cats is incumbent upon understanding the epidemiology of emerging *E. coli* resistance. The purpose of this study is to define the role of integrons and their associated gene cassettes in mediating antimicrobial resistance in canine and feline pathogenic *E. coli*. In this study, we build on previous investigators' findings (Grape *et al*., 2005; van Essen-Zandbergen *et al.*, 2007) by describing the distribution of class 1 and 2 integrons among clinical *E. coli* isolates associated with disease in dogs and cats, and associating the different the types of integrons with their antimicrobial resistant gene cassettes

Materinals and methods

Bacterial isolates and culture conditions

E. coli isolates were collected from dogs or cats with spontaneous infection between May and September 2008. All isolates had been submitted to the contributing

laboratory by veterinary practitioners. The isolates were collected throughout the US. Each isolate was identified as *E. coli* by the submitting laboratory prior to submission to the Clinical Pharmacology Laboratory (CPL) at Auburn University. Upon receipt by the CPL, isolates were plated on CHROMagar™ Orientation (Becton, Dickinson, Franklin Lakes, NJ) to allow for rapid confirmation.

Susceptibility testing

Isolates also were streaked on trypticase agar (Becton Dickinson, Franklin Lakes, NJ) and then incubated at 35°C for 18-24 h. For standard susceptibility testing, cells were harvested and suspended in 4.5 ml of 0.9% normal saline and adjusted to 0.5 McFarland standard turbidity (~10^8 CFU) using SENSITITER® Nephelometer (TREK Diagnostic Systems, Cleveland, OH) before testing. Antimicrobial susceptibility testing was performed using custom made broth micro-dilution susceptibility plates (TREK Diagnostic Systems, Cleveland, OH) according to Clinical and Laboratory Standards Institute (CLSI) guidelines and interpretive standards (CLSI, 2008). Panels of 13 antimicrobial agents were tested (Table 1) including ampicillin, amoxicillin/clavulanic acid, cephalothin, cepfoxitin, cefpodoxime, cefotaxime, ceftazidime, meropenem, enrofloxacin, gentamicin, doxycycline, chloramphenicol, and trimethoprim/sulfamethoxazole. Microdilution plates were incubated at 35°C for 20-24 hours. The MIC values were recorded using the SENSITITER® VIZION system (TREK Diagnostic Systems, Cleveland, OH). For quality control purposes, *E. coli* ATCC® 25922 (American Tissue Cell Culture, Manassas, VA) and *Enterococcus fecalis* ATCC® 29212 (Manassas, VA) were used. Eighty-two of *E. coli* isolates were randomly selected to

represent different phenotypes: single drug resistance (SDR) Phenotype to beta lactams (n=16); SDR phenotype to chloramphenicol (n=3); susceptible or non resistance (NR) phenotypes (n=14); and MDR phenotypes (n=47).

Bacterial DNA preparation, PCR assays and DNA sequencing

Bacterial DNA for PCR reaction was prepared by boiling bacterial cultures in 200 µl of PreMan® Ultra Sample Preparation Reagent (Applied Biosystem, Foster City, CA) for 10 min, followed by centrifugation. The variable regions (i.e. 5'CS and 3'CS) and integrase genes (i.e. *Int1* and *IntII*) of class 1 and 2 integrons were amplified with a PCR reaction using primers listed in Table 2. All primers were synthesized by Operon, Inc. (Huntsville, AL). Amplification reactions were carried out in 25 µl reaction of Omnimix (Cepheid) including 3U TaKaRa hot start Taq polymerase, 200 µM dNTP 4 mM MgCl2 25 mM HEPES buffer pH 8.0± 0.1, 1 ul of DNA preparation, and 1 ul of the forward and reverse primers. Distilled water was added to bring the final volume to 25 µl. The PCR conditions for class 1 and 2 integrons are delineated in Table 2. The reaction products were analyzed by electrophoresis in agarose gels stained with ethidium bromide, and visualized under UV light. The image was recorded using a gel imaging system (Syngene, Frederick, MA, USA). For each set of PCR reactions, serovar Typhimurium DT104 was included as a positive control for class 1 integrons.

Characterization of class 1 and 2 integrons by sequencing and restriction fragment–length polymorphism (RFLP)

RFLP was used to differentiate different integrons fragment similar in size and based on restriction patterns generated by restriction enzymes. One representative from each RFLP type (i.e. having similar restriction patterns on the RFLP) was sequenced, while unique integrons with different size were sequenced as well. The PCR amplicons were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, Calif.) and were sequenced using ABI automatic DNA sequencer (Model 377; Perkin-Elmer) at the Genomic Sequencing Laboratory (Auburn University, Auburn, AL, USA) using the above-described forward and reverse primers, plus the internal, designed primers. DNA sequences were analyzed and compared to published sequences by searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service (http://www.ncbi.nlm.nih.gov/blast/).

Typing of class 1 and class 2 integrons were performed by RFLP. For RFLP analysis, the purified PCR amplicons of variable region of class 1 and 2 integrons were digested using *Alu*I and *HaeIII* restriction endonucleases, respectively, as described (Machado *et al.*, 2005; Machado *et al.*, 2008). Digestions were performed according to the manufacturer's instructions.

Plasmid extraction was carried out on integrons positive isolates using the mini-scale alkaline lysis method using the Qiagen miniprep with some modification. Pulsed-field gel electrophoresis (PFGE) was performed to determine DNA fingerprinting profiles of plasmids for the cured and non cured isolates and to estimate the size of the plasmids

that may carried by class 1 integrons. Plasmid samples (17 µl) were loaded onto a 1% agarose-Tris buffer gel (SeaKem Gold Agarose; BioWhittaker Molecular Applications, Rockland, ME), and PFGE was performed with a CHEF Mapper XA apparatus (CHEF Mapper XA; Bio-Rad Laboratories, Hercules, CA). DNA was electrophoresed for 9 hours at a constant voltage of 200 V (6 V/cm), with a pulse time of 6.75 to 21.7 s, an electric field angle of 120^{o}, and a temperature of 14°C, before being stained with ethidium bromide.

Plasmid-curing experiments

Curing experiments were performed for all integron positive isolates (n=23) using natural curing. Curing was carried out by overnight growth at lower temperature (i.e. 10°C) in trypticase soy agar (TSA) slant without antibiotics. Subcultures were done on TSA slant every 24 hours and the colonies were tested for antimicrobial susceptibility as previously described. Because curing was unsuccessful despite several weeks of subculture, the sodium dodecyl sulfate (SDS) method of curing was implemented on 11 isolates representing both class I and 2 integrons (Tomoeda *et al.*, 1968). An overnight culture of *E. coli* was diluted in Brucella broth (with 10% SDS) and was shaken at 37 C or 40 C for 72 h. Cells were checked for their susceptibility to all drug classes. Only 3 out of the 11 isolates loss their plasmid by curing. The MICs for the cured cells were compared with the MIC before curing to all five drug classes to investigate the contribution of the integrons into the emergence of MDR phenotypes.

Statistical analysis

Susceptibility data were compared from integron-positive and -negative *E. coli.* A statistical comparison of the frequencies and the association of integron presence in *E. coli* was conducted by using odds ratios and 95% confidence intervals. Significance (*P*-value), in terms of the number of resistant and susceptible integron-positive and -negative isolates, was calculated by the Pearson χ^2 -square test ($P < 0.05$). Analyses were performed using the statistical software MINITAB® 15 package (Minitab Inc., PA, USA).

Results

Presence of integrons and the association between integrons and antimicrobial resistance

Of the 82 *E. coli* isolates tested, 27% (n= 22) carried class 1 or 2 integrons. Class 2 integrons where found only in 2.4% (n= 2) isolates. One of these isolates also carried a class 1 integrons. Of the resistant phenotypes (n=65), 34% (n= 22) were positive for either a class 1 and 2 integrons. The association between antimicrobial resistant profile and occurrence of integrons is shown in Figure 1 and Table 3.

All integron positive isolates were characterized by resistance to two or more of antimicrobial agents compared with 56.6% (34/60) for integron negative isolates. All four isolates resistant to all 13 antimicrobial agents carried a class 1 integron (Figure 1).

A significant association was found between the presence of integrons and resistance to cefotaxime, ceftazidime, meropenem, and trimethoprim-sulfamethoxazole (Table 3).

The association between integrons positive isolates and MIC

Among beta lactams, the percentages of resistance were high for the integron positive group; 62%, 77%, 87%, and 83% were resistant to amoxicillin/clavulanic acid, cefotaxime, ceftazidime, and meropenem, respectively, compared with 38%, 23%, 12.5% and 17%, respectively for integron-negative group (Table 3). No differences were detected in the proportion of integron-positive and integron-negative isolates expressing resistance to ampicillin. However, integron-positive isolates were associated with a lower proportion of resistance for cephalothin compared to integron-negative isolates (i.e. 35% versus 65%), respectively.

At least eight fold differences in the MIC_{90s} were observed for beta lactams between integron-positive and integron-negative isolates except for ampicillin. The MIC_{90s} for integron-positive isolates exceeded the MIC breakpoint for all other drug classes (data not shown). In contrast, for integron negative isolates, the MICs were below the susceptible break points (MIC_{90}= 4 μg/ml) for both gentamicin and trimethoprim/sulfamethoxazole. Furthermore, the percentage of isolates resistant to gentamicin and trimethoprim/sulfamethoxazole among integron-positive isolates was significantly high with 65% and 68%, respectively, compared with 35% and 32%, respectively for integron-negative group.

Characterization of gene cassettes associated with class 1 and 2 itegrons

A total of 11 gene cassettes (Table 4) were identified by PCR amplification of the conserved regions of class 1 integron. The size of PCR product varied from 1 kb to

3.2 kb. The integrons were found to contain one to three gene cassettes and to differ in the orders of these gene cassettes. RFLP characterization of class 1 revealed 7 distinct profiles of gene cassette arrays. These were *aadA1, dfrA1-aadA1, dfrA17-aadA5, dfrA12-aadA2, aadB-aadA1d, aacA4-catB3-dfrA1*and *aadB-aadA1-cmlA6*. Among these profiles, the gene cassette *aadA1* was found most frequently (65.2%, n= 15/23) in class 1 integrons either alone or in combination with other gene cassettes. These gene cassettes encoded for resistance to certain antimicrobial agents, including *aadA1*, *aadA2*, and *aadA5* (which confers resistance to streptomycin and spectinomycin), *aacA4, aadB* (which confers resistance to aminoglycosides), the dihydrofolate reductase gene cassettes *dfrA1* and *dhfrA17*, *dfrA12, dhfr1* (which confer resistance to trimethoprim), *catB3, cmlA6* (which confers resistance to chloramphenicol) and *sat1* (which confers resistance to streptothricin). The antimicrobial resistance gene cassettes found in class 1 and 2 integrons correlates with the corresponding antimicrobial phenotypes found in those isolates (Table 4).

A BLAST search for the published sequence in GenBank revealed the following high similarities for the most prevalent gene cassettes found in *E. coli* isolates from different geographical locations and sources (Table 5).

The two isolates with class 2 integrons contained the cassette array *dfrA1-sat1-aadA1*; the class 1 gene cassette array found in one of the isolates was *aacA4-catB3-dfrA1*.

Curing experiments for isolates carrying plasmids harboring class 1 integrons.

Curing experiments of plasmids carrying class 1 integrons occurred successfully in the following 3 isolates (gene cassettes): L8055239 (*aadA1*), I3195059 (*aacA4-catB3-dfrA1*), and B5729897 (*aadB-aadA1-cmlA6*). Class 1 integrons were detected in the extracted plasmid DNAs among those isolates before but not after curing using PCR protocol (Figure 2). PFGE revealed that several cured isolates lost 2.3-kb, 23-kb, 97-kb, 145.5-kb, 169-kb, and 194-kb bands after curing (Figure 2). All *E. coli* cured isolates become susceptible to the antimicrobials for which the integron's gene cassette array encoded resistance (Table 6). Interestingly, some isolates become susceptible to some beta lactams including ampicillin, cefotaxime, ceftazidime, and ticarcillin/clavulanic acid. None of the gene cassettes identified within the integrons from those isolates confer resistance to beta lactams.

Discussion

The increase in the incidence of antimicrobial resistance among canine and feline *E. coli* isolates has been reported in many countries. However the mechanism of acquisition and dissemination of antibiotic resistance genes in clinical *E. coli* isolates from dogs and cats is not well understood, particular in the US. In our study, we documented the prevalence of class 1 and 2 integrons among clinical samples of *E. coli* collected from dogs and cats with spontaneous infectious in the US. Additionally, different types of class 1 integrons were characterized which carried different gene cassettes. Furthermore, class 2 integrons were reported for the first time among canine

and feline clinical *E. coli* isolates in the US. To our knowledge, this is the first report that investigates the distribution and to characterization of class 1 and 2 integrons among clinical specimens of *E. coli* from dogs and cats throughout the US.

In the present study, the incidence of class 1 integrons among canine and feline clinical samples (27%)was similar to the data found in Switzerland, in which 22% of the isolates from companion animals carried class 1 integrons (Cocchi *et al.*, 2007). However, many studies have documented a higher proportion of class 1 integrons among *E. coli* clinical samples from farm animals. It was found that 52% of the strains isolated from farm animals carried class 1 integrons (Cocchi *et al.*, 2007). Other studies indicated an even higher prevalence of class 1 integrons in *E. coli* from farm animals: 59% reported in calf diarrhea cases (Du *et al.*, 2005), 63% in isolates from chicken (Bass *et al.*, 1999), 64.4% reported in swine with diarrhea (Kang *et al.*, 2005b), and 82% in isolates from chicken (Keyes *et al.*, 2000). The differences between our results and those found in the other reports may reflect the variations with selection pressure in different environments (Rosser and Yound, 1999). Thus, the *E. coli* strains food production animals (raised for economic purposes) appear to be more frequently exposed to antimicrobial pressure compared with companion animals (Cocchi *et al.*, 2007).

In this study we have also reported a very low percentage of isolates that carried a class 2 integron (only two isolates). Although other studies have reported a higher proportions of class 2 integrons in other species, the proportion of class 2 integrons appears to be lower in comparison to class 1 integrons: 14% versus 92% (Goldstein *et al.*, 2001), 9.5% versus 55.2 (Grape *et al.*, 2005), and 6% versus 12% (Sunde, 2005) of the

isolates that were found to carry class 2 and 1, respectively. Furthermore, in contrast to class 1 integrons, for which 11 different gene cassettes have been identified, only 3 different gene cassettes have identified (i.e. *dhfr1- sat1- aadA1*) in class 2 integrons.

Similarly the gene cassette array *dhfr1- sat1- aadA1* was detected most frequently in *E. coli* isolates from dogs and cats from Germany (Kadlec and Schwarz, 2008) and in *E. coli* from animal and human sources (Kang *et al.*, 2005a; van Essen-Zandbergen *et al*., 2007). Only six gene cassettes have been associated with class 2 integrons (Ramírez *et al.*, 2005; Mazel, 2006). Thus this reduction in the diversity and prevalence of class 2 integrons compared to class I integrons may suggest that the gene encoding integrase (*IntI2*), which catalyzes the recombination events between primary recombination site (*attI*) and a secondary target site (attC site), contains nonsense mutations in codon 179, producing a non functional pseudogene (Hansson *et al.*, 2002; Fluit and Schmitz, 2004; Mazel, 2006).

Class 1 integrons were significantly associated with resistance to all the antimicrobials tested in this study. However, integron positive strains were significantly higher among isolates with resistant to beta lactams (e.g. cefotaxime, meropenem), aminoglycosides (e.g. gentamicin) and potentiated sulfanomide (e.g. trimethoprim/sulfamethoxazole). More than 80 gene cassettes, which cover resistance to different drug classes in gram negative and positive bacteria are associated with class 1 integrons (Mazel, 2006). Our study characterized gene cassettes that confer resistance to aminoglycosides, including *aadA1, aadA2 aadA5, aacA4* and *aadB*, and genes that confer resistance to trimethoprim, including *dfrA1*, *dhfrA17* and *dfrA12*. The most common

detectable gene cassette is the resistant gene (Kang *et al.*, 2005a), which encodes for an aminoglycoside adenyltransferase that confers resistance to streptomycin-spectinomycin. A previous study indicated that the presence of a single antibiotic resistance gene, *aadA1*, was most prevalent among *E. coli* isolates from veterinary sources (Goldstein *et al.*, 2001). This antibiotic resistance gen was detected most frequently, either alone or in combination with other gene cassettes, in the *E. coli* isolates from dogs and cats in Germany (Kadlec and Schwarz, 2008). Other studies indicated that *aadA1* was detected more frequently in the *E. coli* isolates from human and animal sources in Netherland (van Essen-Zandbergen *et al.*, 2007) and from environmental sources (Moura *et al.*, 2007). Furthermore, our study is the first to observe the presence of *aacA4-catB3-dfrA1*in US *E. coli* from companion animals. These resistance genes confer resistance to chloramphenicol

The gene cassettes characterized in the present study showed high identity to those gene cassettes found in same or different bacterial species, either from animals or humans, and distributed among different geographical location. This data suggests that class 1 integrons may contribute significantly to the horizontal transfer of antimicrobial resistance genes among bacterial species from different sources or geographical locations (Chang *et al.*, 2000; Yu *et al.*, 2003). However, the gene cassettes observed in class 1 and class 2 integrons did not correlate with the phenotypes that were observed in canine and feline clinical *E. coli* isolates. It is likely that other mobile genetic elements (and mechanisms such as efflux pump or modifications of porins contribute to the emergence of multi-drug resistance in these clinical isolates (Bass *et al.*, 1999; Piddock, 1999).

The process of plasmid curing was more effective when using 10% SDS compared with natural curing at low temperature. The mechanism of SDS action is not completely understood. However, their effectiveness as anionic detergents can cause disruption of cell membranes - completely or partially - for which plasmid replicons were more closely associated (attached) with it, therefore damaging the plasmid by SDS (El-Mansi *et al.*, 2000). This leads to imperfect replication or unsuccessful segregation of plasmid. Consistent with a previous study, the curing was not completely efficient for these *E. coli* isolates (Tomoeda *et al.*, 1968). It is possible that plasmid may survive by integration into the genome, even with the absence of selective pressure (El-Mansi *et al.*, 2000). Furthermore, we found that some isolates lost other resistant determinants after curing (i.e. beta lactam drugs) which is not readily carried by class 1 integrons (Table 6). This may be attributed to losing more than the gene cassette that is different from that found in class 1 integrons which is either carried in the same or in different plasmids. The data highlights the importance of other genetic elements, besides integrons, in horizontal gene transfer of resistance genes.

The present study demonstrates the emergence of class 1 and 2 integrons among clinical isolates of *E. coli* from dogs and cats throughout the US. To our knowledge, this is the first report of the presence of class 2 integrons among clinical *E. coli* isolated from canine and feline in the US. The proportion of isolates carrying class 1 and 2 integrons is not significant as found in food production animals and humans, which may be attributed to different antimicrobial pressures for isolates from companion animals. The gene cassettes characterized in the class 1 and class 2 integrons might reflect the selective

pressures resulting from different antimicrobial therapy, drugs used, and the regions from which the isolates originated. Therefore, this study can provide initial information for a surveillance program aimed at the prudent use of antimicrobial agents by veterinarians in the US.

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Table 1. Antimicrobial drugs used in this study

Drug class	Antimicrobial drugs	MIC_{BP} (S/R, mg/L)[a]	MIC range (mg/L)[b]
Beta lactams	Ampicillin	≤8, ≥32	0.5–256
Penicillins	Amoxicillin/ clavulanic acid	≤8/4, ≥32/16	0.25–512
	Meropenem	≤4, ≥16	0.25–512
	Ticarcillin/ clavulanic acid	≤16/2, ≥128/2	2–2048
Cephalosporins	Cefpodoxime	≤2, ≥8	0.12–128
	Cefotaxime	≤8, ≥64	1–1024
	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	0.5–1024
Macrolides	Azithromycin	≤0.5 , ≥8	0.12–256
Aminoglycosides	Gentamicin	≤4, ≥8	0.12–256
Potentiated sulfa	Trimethoprim/ sulfamethoxazole	≤2, ≥8	0.06–128

MIC, minimum inhibitory concentration; BP, break point; S, susceptible break point; R, resistant break point.

[a]The MIC beakpoints were designated according to CLSI guidelines and interpretive standards (CLSI, 2008). [b]The MIC ranges for custom-made micodilution plates.

Table 2. Primers sequences and PCR conditions used in this study

Primer	Oligonucleotide sequence (5' to 3')	PCR conditions	Reference
IntI1-F	GGT CAA GGA TCT GGA TTT CG	1 cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C; 1 cycle of 8 min at 72°C	(Machado *et al.*, 2005)
IntI1-R	ACA TGC GTG TAA ATC ATC GTC		(Machado *et al.*, 2005)
5'CS	GGC ATC CAA GCA GCA AG	1 cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 66°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	(Levesque *et al.*, 1995
3'CS	AAG CAG ACT TGA CCT GA		(Levesque *et al.*, 1995
IntI2-F	CAC GGA TAT GCG ACA AAA AGG T	Same as for *int1*	(Machado *et al.*, 2005)
IntI2-R	GTA GCA AAC GAG TGA CGA AAT G		(Machado *et al.*, 2005)
attI2-F	GAC GGC ATG CAC GAT TTG TA	Same as for 5'CS	(Machado *et al.*, 2005)
orfX-R	GAT GCC ATC GCA AGT ACG AG		(Machado *et al.*, 2005)

Figure 1. Association between antimicrobial resistant profile and occurrence of class 1 and 2 integrons in *Escherichia coli* stains isolated from dogs and cats.

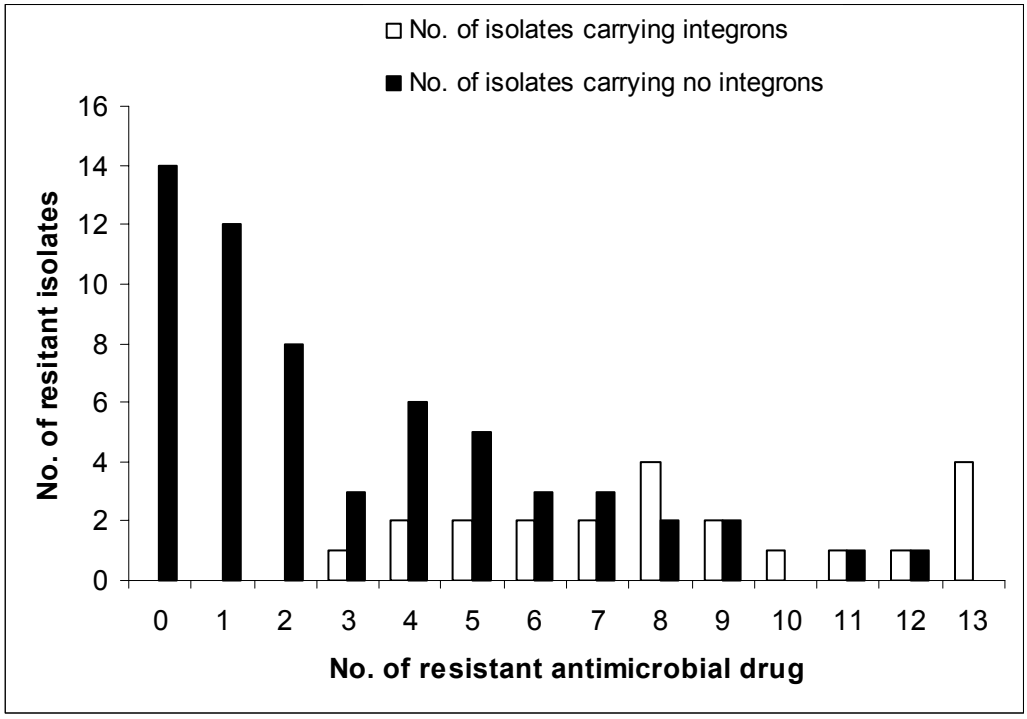

Table 3. The association between antibiotic profile and class 1 integrons in *E. coli*

Antimicrobial drug	**Integrons-positive isolates (n=23)**		**Integrons-negative isolates (n=59)**		
	%R (n)[b]	**%S (n)**	**%R (n)**	**%S (n)**	**OR (95% CI)**
Ampicillin	50(19)	7(3)	50(19)	93(40)	13.3 (3.51-50.64)
Amoxicillin/ clavulanic acid	62(13)	14(8)	38(8)	86(51)	10.3 (3.26-32.84)
Cephalothin	35(20)	8(2)	65(37)	92(22)	5.9 (1.27-27.91)
Cepfoxitin	59(13)	15(9)	41(9)	85(50)	8.0 (2.65-24.28)
Cefpodoxime	58(11)	18(11)	42(8)	82(51)	6.4 (2.08-19.54)
Cefotaxime	77(10)	18(12)	23(3)	82(56)	15.5 (3.71-65.19)
Ceftazidime	87.5(7)	20.5(15)	12.5(1)	79.5(58)	27 (3.08-237.25)
Meropenem	83(5)	23(17)	17(1)	77(58)	17 (1.86-156.13)
Enrofloxacin	58(11)	18(11)	42(8)	82(51)	6.4 (2.08-19.53)
Doxycycline	50(17)	11(5)	50(17)	89(42)	8.4 (2.67-26.40)
Chloramphenicol	48(14)	15(8)	52(15)	85(44)	5.1 (1.80-14.64)
Gentamicin	65(11)	28(11)	35(6)	72(53)	8.8 (2.69-28.97)
Trimethoprim/ sulfamethoxazole	68(15)	11.5(7)	32(7)	88.5(52)	16 (4.82-52.57)

[a]MICs were determined using microdilution methods according to CLSI standards (CLSI, 2008).

[b] R, resistant; S, susceptible.

Table 4. Antibiotic resistance patterns of *E. coli* isolates and their relationship with occurrence of the integrons

RFLP type	Length of variable region (bp)	Gene cassettes and order	Resistance phenotype[a]	No. of isolates	Species	Tissue source	US State (n)[b]
Class 1 integrons							
I_1	1000	*aadA1*	XACDEGR	1	Feline	Urine	CA (1)
II_1	1500	*dfrA1-aadA1*	XACS	1	Canine	Urine	NC (1)
VI_1	1500	*dfrA17-aadA5*	ACDGRERS	2	Canine	Urine	MA (1), NC (1), CA (1)
III_1	1800	*dfrA12-aadA2*	HDS	1	Canine	Urine	CA (1)
	1500	*aadB-aadA1d*	ACDG	1	Canine	Urine	CA (1)
	2500	*aacA4-catB3-dfrA1*	XATOPZCHDERSGM	4	Canine	Urine, Tracheal wash	OH (1), NC (2), IL (2)
	3200	*aadB-aadA1-cmlA6*	AHDGSXTOPZCER	2	Canine	Urine	CA (2)
	2000	*aadA1-unknown*	XATOPCHDEMGRSZ	9	Canine, Feline	Urine, Nasal cavity, Vagina	IL (2), CA (5), OH (1), NC (1)
Class 2 integrons							
II_2	2200	*dhfr1-sat1-aadA1*	XATOPZCHDEGRS	2	Feline, Canine	Urine	NC (2)

[a] Abbreviation of antimicrobial drugs; A, ampicillin; X, amoxicillin/clavulanic; C, cephalothin; O, cepfoxitin; P, cefpodoxime; T, cefotaxime; Z, ceftazidime; M, meropenem; E, enrofloxacin; D, doxycycline; H, chloramphenicol; G, gentamicin; S, trimethoprim/sulfamethoxazole. [b] Abbreviation of the origin of the isolates; CA, California; NC, North Carolina; IL, Illinois; MA, Massachusetts; OH, Ohio.

Table 5. A BLAST search for the antimicrobial gene cassettes

The gene cassette array	Source	Accession number	% of identity
dhfr1- sat1- aadA1	Japan	(AB188272)	100%
	Germany	(AM990340)	100%
	Portugal	(EU089672)	99%
	Turkey	(EU339237, EF543147)	99%
	China	(AB211124)	99%
	Ireland	(FJ155843)	99%
dfrA17-aadA5	Turkey	(FJ001849, FJ001848, FJ001847)	99%
	Netherlands	(DQ663488)	99%
	Japan	(AB188265, AB188260)	99%
	Germany	(AM932673)	99%
aacA4-catB3-dfrA1	China	(DQ836057, EF488369)	99%
	Germany	(AM939644)	99%
aadB-aadA1-cmlA6	China	(DQ836058, DQ018384)	99%

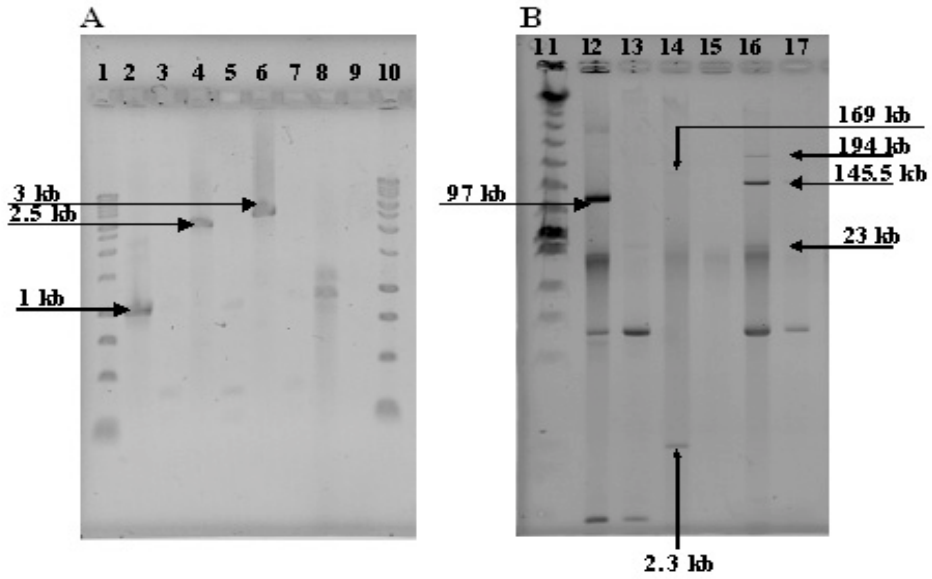

Figure 2. PFGE profiles of the extracted plasmid DNA of the 3 isolates before and after curing.

PCR amplification, A, and PFGE profiles, B, for the isolates before and after curing. Lanes 2, 4, 6, PCR positive result for the variable regions of class 1 integrons for isolates L8055239, I3195059, and B5729897 with corresponding amplicons size 1-kb, 2.5-kb, and 3.2-kb, respectively before curing. Lanes 3, 5, 7, negative results of class1 integrons for isolates L8055239, I3195059, and B5729897, respectively. Lanes 8 and 9 represent isolates *salmonella* serovar Typhimurium DT104 and *E. coli* ATCC® 25922, respectively used as positive and negative control for class 1 integrons. Lanes 12, 14, 16, isolates L8055239, I3195059, and B5729897, respectively before the curing. Lanes 13, isolate L8055239 lost a 97-kb band; lanes 15, isolate I3195059 lost a 169-kb and 2.3-kb bands; lanes 17, isolate B5729897 lost a 194-kb, 145.5-kb and 23-kb bands after curing.

Table 6. Antimicrobial resistance profiles and MICs for the isolates before and after curing.

	Bacterial isolate(s)					
Antimicrobial drugs	L80552 39 (B)	L80552 39 (A)	I31950 59 (B)	I31950 59 (A)	B57298 97 (B)	B57298 97 (A)
amoxicillin/ clavulanic acid	16	8	64	16	8	1
ampicillin	**> 256**	**4**	**>256**	**4**	**>256**	**≤0.5**
cefotaxime	≤1	≤1	**16**	**≤1**	≤1	≤1
cepfoxitin	8	8	128	16	2	2
ceftazidime	≤0.5	≤0.5	**64**	**2**	≤0.5	≤0.5
cephalothin	256	32	>1024	32	**16**	**2**
chloramphenicol	4	8	**1024**	**8**	**32**	**8**
doxycycline	32	32	64	32	16	8
enrofloxacin	64	64	>128	64	1	1
gentamicin	**128**	**1**	**8**	**1**	**128**	**2**
meropenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
tricarcillin/ clavulanic acid	**256**	**4**	**256**	**8**	16	≤2
trimethoprim/ sulfamethoxazole	0.25	0.25	**>128**	**≤0.06**	**>128**	**0.12**

[a] MICs were determined using broth microdilution methods according to CLSI standards (CLSI, 2008).

Numbers in boldface represent resistant phenotypes that becomes susceptible after curing. Only the curing of isolate B5729897 resulted in susceptible phenotype