Fluoroquinolone-associated mutations in *soxS*, a transcriptional regulator of AcrAB efflux pump

by

Caterina Isabella Lazzaroni

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Biomedical Science

Auburn, Alabama
December 14, 2013

Keywords: antimicrobial resistance, fluoroquinolones, Escherichia coli

Approved by

Dawn M. Boothe, Chair, Professor of Physiology and Pharmacology
Robert Judd, Associate Professor of Physiology and Pharmacology
Stuart Price, Associate Professor of Pathobiology

Abstract

Escherichia coli is a major cause of urinary tract infections in companion animals and efflux pump over expression has been associated with high-level fluoroquinolone (FQ) resistance. The purpose of this study was to evaluate the impact of *in vitro* exposure of different generations of fluoroquinolones on the mutability of *soxS* in clinical canine *E.coli* isolates and to evaluate the impact of these mutations on the expression of AcrAB and EmrE efflux pumps. Broth macrodilution was performed to expose SDR, NDR, MDR and ATCC isolates to 2-64 X their FQ MIC for 30, 60, 90 and 120 minutes. Amplification and sequencing of *soxS* revealed the presence of three novel mutations *(*M78K, Q56K, L59R) when exposing SDR, NDR and ATCC isolates to 0.06 and 0.12µg/mL of ciprofloxacin for 30, 60 and 90 minutes. No mutations were identified in isolates exposed to newer generation FQs. Overexpression of *acrB* was identified at concentrations and time points that had previously induced *soxS* mutations. An increase, but not overexpresssion occurred in *emrE* of ciprofloxacin exposed isolates. This study will contribute to the accumulated knowledge regarding mechanisms whereby fluoroquinolones cause MDR, thus providing evidence for selecting one antimicrobial over another.

Acknowledgments

I would first like to thank God who granted me the privilege to pursue this program, without His guidance and my eternal faith in Him, I would not have been able to reach this milestone.

I would also like to express my most sincere gratitude to all those who have contributed, both personally and professionally, to this thesis. In particular, I would like to thank:

Dr. Dawn Boothe for opening the doors of her laboratory and taking me in as a Master's student. I will be forever grateful for the support you gave me then and have continued to give me throughout the program. It has been a pleasure and an honor working under your wing.

Dr. Thungrat, Dr. Gossett and Jameson Sofge for making the Clinical Pharmacology Lab a wonderful place to work at. I sincerely appreciate all the kind words, support and advice that you have given me throughout. My next work colleagues will have big shoes to fill!

Crisanta Cruz for providing me with a sense of home through your words and kindness. Talking to you in Spanish would always make my day. Thank you for your support and your invaluable advice. La quiero mucho!

Dr. Judd for your guidance and kind words. Thank you for all the support you have given me since day one.

My friends, Grupo Niche, for your words of encouragement throughout and for your priceless friendship. You guys have certainly made my stay in Auburn memorable. Love y'all!

Vesko, for your understanding, support and love through the years. Thank you for being by my side to remind me to believe in myself. Love you.

Finally, my family. Words will always fall short when trying to express my gratitude towards them. Thank you for providing me with enough love to endure time and distance. Your love and belief in me has always been my propeller. This thesis is dedicated in its entirety to my family.

Table of Contents

List of Tables

List of Figures

CHAPTER I
Literature Review

Antimicrobials

Penicillin, the first antibiotic, was discovered by Alexander Fleming in 1929 when he accidentally noticed that staphylococci growth was inhibited by a *Penicillium* mold. Sir Alexander Fleming warned that the inappropriate use of penicillin could lead to the selection of resistant ‘‘mutant forms’’ that could cause more serious infections and thus could pass a resistant version of the microbe. Interestingly, within one year of the widespread use of penicillin a significant number of strains of this bacterium had become resistant to penicillin and only a few years later, over 50% were no longer susceptible to this new drug (Levy, 2002). Currently, literature suggests that “for each class of antimicrobial drugs approved for use in human medicine, resistance has generally emerged within 1 to 2 decades of use” (Boothe, 2012).

The concept of antibiosis began when Alexander Fleming discovered lysed colonies of *Staphylococcus sp.* on a plate contaminated with penicillum mold. The term “antimicrobial” is defined as the substance of natural, semisynthetic, or synthetic origin that kills or inhibits the growth of a microorganism but causes little or no damage to the host (Walsh, 2003).

Antimicrobial drugs can be classified by various criteria, including but not limited to,chemical structure, spectrum of activity and mechanism of action. The mechanism of action of each drug determines drug efficacy and mechanisms of resistance.. Based on mechanism of action, antimicrobials are generally divided into 4 main categories: (1) inhibition of cell wall

synthesis, (2) inhibition of protein synthesis, (3) inhibition of nucleic acid synthesis, and (4) inhibition of folic acid biosynthesis (Boothe, 2012)..

Multiple proteins located in the bacterial cell wall are important in cell wall synthesis during division and growth of organisms. This important role in bacterial growth renders these proteins are the target of several antimicrobial agents. The destruction of the peptidoglycan layer, which provides additional support to the cell wall, results in osmotic lysis of the cell. Ribosomes play an important role in protein synthesis. Antimicrobials that target ribosomes and can either inhibit protein formation or cause formation of defective proteins resulting in detrimental effects to the bacterial organism. Another target of antimicrobials is the nuclear material. Targeting cellular DNA and interfering with cellular DNA synthesis generally results in cell death. Lastly, antimicrobials may also target important metabolic pathways such as folic acid synthesis. By interfering with such pathways, the ability of the microorganism to produce vital materials is impaired (Boothe, 2012).

Antimicrobial resistance

Appreciation of antimicrobial mechanisms of action allows for the identification of mechanisms by which antimicrobial resistance might be avoided or minimized.

Antimicrobial resistance emerges when the therapeutic concentrations of an antimicrobial fail to effectively kill or inhibit the growth of the entire population of the targeted organism. The development of such resistance becomes a medical concern since it can lead to therapeutic failure in the patient. Two elements are necessary for antimicrobial resistance to develop: the

presence of an antimicrobial capable of preventing growth of the majority of bacteria present and a heterogeneous colony of bacteria where at least one of these bacterium carries the genetic determinant capable of expressing resistance to the antibiotic (Levy, 2004). The bacteria that are able to survive the presence of the antimicrobial code for the genetic information necessary to survive antibiotic stress and they are now capable of propagating these selected genes.

Acquired Antimicrobial Resistance

Susceptible bacteria can acquire selected resistance by accepting antimicrobial resistance genes from resistant bacteria. Acquired resistance reflects the ability of bacteria to incorporate extra chromosomal DNA carrying resistant factors (Boothe, 2012).Transfer of genetic determinants can be carried on plasmids, bacteriophages, transposons, and other mobile genetic material. Extra-chromosomal DNA (i.e., plasmids and bacteriophages) encode for resistance and can be transmitted horizontally (from resistant to susceptible bacteria) and even across species and genera. Acquired resistance results from successful gene change and/or exchange that involves horizontal gene transfer via transformation, transduction or conjugation.

Conjugation is probably the most important and common mechanism of horizontal transfer. When bacteria are in close proximity, a hollow tubular structure known as pilus temporarily connects two bacteria. One bacteria will behave as a donor, while the other is the recipient. Plasmids, which can replicate independently from the chromosome, are transferred from one bacterial cell to the other through the formation of the pilus and thus transmission of

resistant genes takes place. In order to allow successful transfer of genetic material, close contact between donor and recipient is needed (Schwarz, 2001).

In transduction, the virus that contains the genes for antibiotic resistance infects a new bacterial cell and introduces its genetic material into the receiving bacteria. The infecting bacteriophage also introduces its own viral DNA into the host's genome and thus forces the bacteria to produce more copies of this infectious bacteriophage. Copies will continue to multiply until the bacteria dies and liberates new bacteriophages that will go on and infect other cells (Schwarz, 2001).

A much "simpler" type of gene transfer occurs through transformation. Transformation allows for the bacteria to uptake DNA that usually originates from cells that have died in the vicinity. This "naked DNA" is simply incorporated into the genome of the bacteria If antimicrobial resistance genes are released by dead bacterium, they may be taken and incorporated in the genome of nearby bacteria (Alanis, 2005).

Determining Antimicrobial Susceptibility versus Resistance

Susceptibility data based on broth dilution procedures that are reported for an existing infection will include MIC (minimum inhibitory concentration) as well as identification of the organism's phenotype (susceptible or resistant).

The MIC is defined as the minimum concentration that needs to be reached in order to inhibit the visible growth of the pathogen of interest. Broth microdilution is particularly advantageous and efficient in determining the drugs the pathogen is susceptible or resistant to

and the particular MIC for the selected susceptible antimicrobials. By performing broth microdilution and determining the MIC, one is able to better understand the interaction between the microbe and the drug (Drlica and Schmitz 2002).

In a 96-well broth microdilution plate, the pathogen of interest is inoculated in each well and each column of wells contains a standard antibiotic concentration that increases by 2-fold when moving from left to right. The MIC is then determined by indentifying the well with the lowest concentration of drug in which where there is no visible growth. The magnitude of the MIC establishes the phenotype (susceptible or resistant) of the pathogen, providing therapeutic guidance. An isolate is considered susceptible if the MIC lies below the breakpoint for that particular antibiotic and resistant when the isolate is able to grow after *in vitro* exposure to a drug concentration that equals the upper threshold or resistant MIC breakpoint (Boothe, 2012). The susceptibility status of each drug is based on comparisons of the MIC for that drug to that of Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility standards as delineated in M100-S18 (CLSI 2008).

Minimum Inhibitory Concentration (MIC) vs. Mutant Prevention Concentration (MPC)

During the last two decades, the MPC has been promoted in lieu of the MIC for designing dosing regimens (Drlica and Schmitz 2002). Boothe defines MPC in her book Small Animal Pharmacology as "the concentration of drug that is necessary to inhibit first-step mutants, or the MIC of the least susceptible isolate in a resident population of pathogens." Targeting the MIC rather than MPC is likely to facilitate the emergence of the subpopulation

mutants. On the other hand, targeting the MPC decreases the risk of their emergence as the predominant population.

When cultured, the MIC reported for the population statistically is the most common MIC (mode) in that population. In normally distributed populations the mode should be equivalent to the median or the MIC_{50} of the population. However, the MIC of the first-step mutant will be at the high end of the population MIC range. This high end value represents the mutant prevention concentration (MPC). Moreover, the mutant selection window (MSW) encompasses the lower threshold represented by the culture MIC and the upper threshold represented by MPC. Drlica (2007) states that the mutant selection window hypothesis maintains that drug-resistant mutant subpopulations present prior to initiation of antimicrobial treatment are enriched and amplified during therapy when antimicrobial concentrations fall within a specific range (the mutant selection window). Therefore, if the targeted drug concentrations lies within MSW, a mutant isolate is likely to emerge. Moreover, when drug resistance is acquired stepwise, the mutant selection window increases, making the suppression of each successive mutant increasingly more difficult.

If treatment targets only the MIC from the culture report, likely to be the MIC_{50}, then only the isolates at or below the MIC will be inhibited. Additionally, the isolates that required a higher MIC than MIC_{50} (isolates growing within the mutant selection window), will continue to expand and a second distribution curve will emerge. These mutants will require a higher MIC and gradually become second-step mutants. Not targeting to inhibit the growth of the entire population leads to the emergence of a second population that becomes resistant to the antibiotic.

In vitro data (Drlica et. al., 2003) has shown that by targeting MPC levels of fluoroquinolones one can indeed inhibit emergence of strains of E.coli isolates that harbor first-step *gyrA* mutations (a mechanism of antimicrobial resistance against fluoroquinolones).

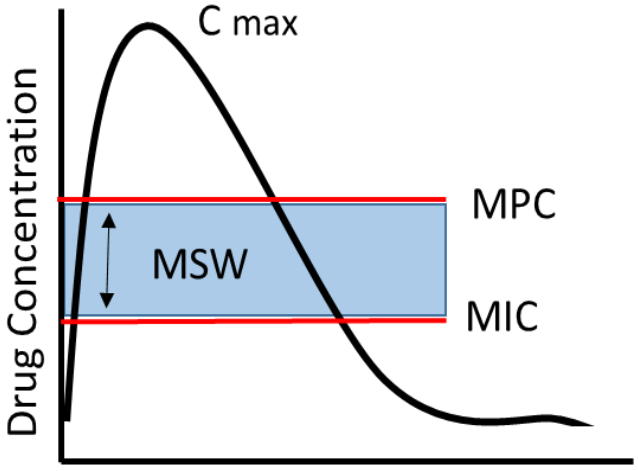


Figure 1. Schematic representing MPC, MIC and MSW

Antimicrobial resistance in *E.coli*

E.coli are gram negative facultative anaerobic bacteria. They are the primary gram negative facultative anaerobe in the gut and they adhere to the lumen of the lower large intestine. *E.coli* are part of the normal commensal inhabitants of the gut and live in a symbiotic relationship with the host. However, *E.coli* become uropathogenic once the gastrointestinal barriers are violated. In ascending infections of uropathogenic E.coli (UPEC), fecal E.coli colonize the urethra, spreading to the urinary tract and bladder.

Boothe (2006) stated that *E.*coli was the pathogen causing 50% of UTI cases present in dogs (n=240) admitted to Auburn Small Animal Teaching Hospital. Seguin (2003) also examined recurrent UTIs in dogs (n=441 isolates) and determined that E.coli was the most common causative pathogen (47%) of recurring UTIs. Boothe (2001) found that more than 50% of uropathogenic organisms were resistant to first-choice drugs such as amoxicillin. Moreover, 40% of organisms were resistant to fluoroquinolones (which is the first choice for complicated infections). Oluoch (2001) states that resistance to fluoroquinolones is most of the time multi-drug in nature. Boothe *et.al* (2005) also demonstrated a high incidence of MDR in uropathogenic *E.coli* isolates from canine patients (n=175) that had been admitted to Auburn University's Small Animal Hospital. The results concluded that 61.5% of isolates that were MDR were also associated with enrofloxacin resistance, indicating that fluoroquinolone-resistant isolates were more likely to be multi-drug resistant (MDR).

Emergence of antimicrobial resistance in *E.coli* isolates from small animal patients presents a concern not only in the realm of animal health, but it also presents health consequences to humans if these isolates are transmitted from their pets (Beutin *et. al*, 1999).

Johnson et.al, 2001 indicated that phylogenetic similarities between *E.coli* isolates from urinary tract infections (UTI) in dogs and extra intestinal pathogenic *E.coli* (ExPEC) infections in humans have been identified. The study also demonstrates that over 15% of canine feces were found to contain *E.coli* strains closely related to human virulent ExPEC colonies. Moreover, resistance mechanisms (i.e. resistance genes) in small animal *E.coli* isolates are the same as those in the resistant strains found in humans (Guardabassi et.al., 2004; Webber and Piddock et .al.,

2001). Studies like the ones mentioned, should encourage veterinarians to understand the importance and the impact of antimicrobials and the emergence of its resistance patterns.

Fluoroquinolones

Fluoroquinolones (FQ) are a large class of synthetic antibacterial drugs. The progentitor of the fluoroquinolones was first nalidixic acid. Its ring structure is 1,8-naphthyridine nucleus that contains two nitrogen atoms. In 1986, ciprofloxacin was approved for use in humans and enrofloxacin was then approved for veterinary use. Fluoroquinolones are classified into generations in order to elucidate the development and chemical manipulation that has occurred throughout the years.Newer generations of fluoroquinolones have been designed in order to broaden antimicrobial spectrum and decrease the risk of resistance (Andriole, 2005).

Current fluoroquinolones consist of a quinolone ring nucleus which contains a carboxylic acid group at position 3 and an exocyclic oxygen at position 4. These two positions are designated as the active DNA gyrase binding sites and therefore these two sites are not chemically manipulated (Boothe, 2012).

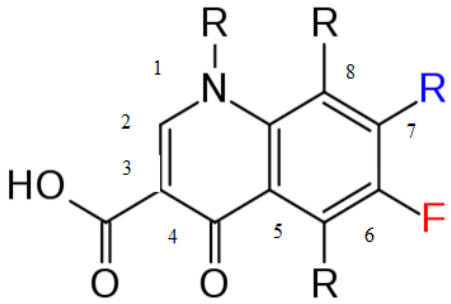


Figure 2. Structure of fluoroquinolone

The introduction of fluorine to position 6 greatly increased the number of targeted gram-positive bacteria. Also, the introduction of the piperazyl ring at position 7, such as in second generation fluoroquinolones, increased the efficacy of the drug to penetrate a variety of tissues (Lu, 2001).

The first generation of fluoroquinolones target Gram negative bacteria and lack antimicrobial activity against Gram positive bacteria. Moreover, first generation fluoroquinolones, such as norfloxacin are able to reach high concentrations in the urinary tract and thus have been used extensively in the treatment of urinary tract infections. However, Ball (2000) explains that first generation fluoroquinolones have poor absorption following oral intake and that poor tolerance limits their clinical use.

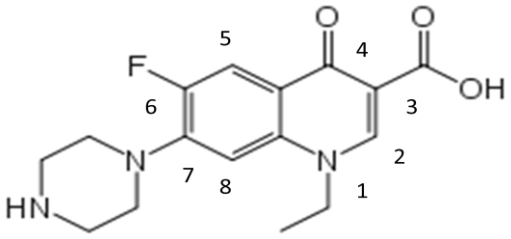


Figure 3. Structure of norfloxacin

Second generation fluoroquinolones were developed by adding a cyclic diamine, piperazinyl or methylpiperazinyl moiety at position 7 and a fluorine atom at position 6 in addition to a carboxy group at C-3 and a keto group at C-4. Second generation fluoroquinolones include a broad spectrum for gram-negative bacteria versus gram- positive bacteria. A number of

fluoroquinolones belong to the second generation including ciprofloxacin, ofloxacin, and enrofloxacin. Enrofloxacin was the first approved fluoroquinolones for dogs, it is structurally similar to ciprofloxacin, it is active as the parent compound and is metabolized to ciprofloxacin in dogs.

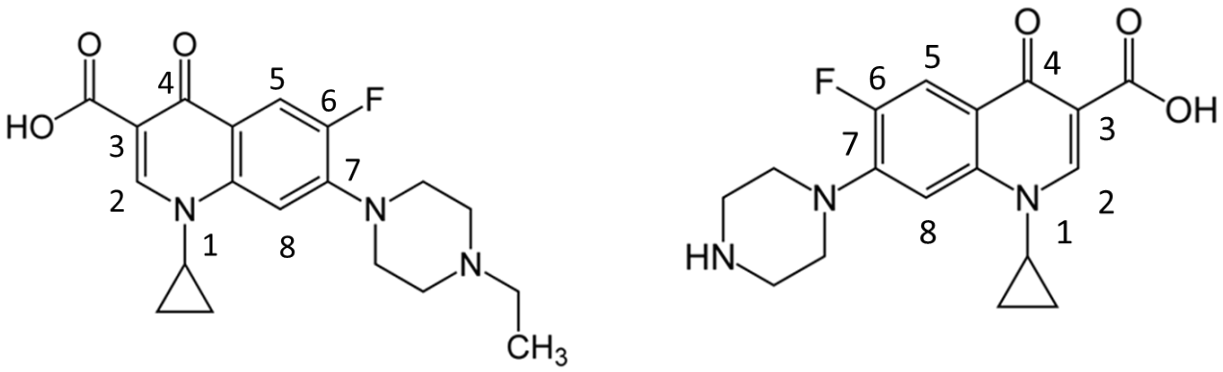


Figure 4. Structure of enrofloxacin (right) and ciprofloxacin (left)

Third generation fluoroquinolones differ from previous generations due to the incorporation of substituents at positions 1, 7 and 8 of the quinolone nucleus. Pradofloxacin is a third generation fluoroquinolone that was developed exclusively for veterinary use. Other third generation drugs include levofloxacin, sparfloxacin, and gatifloxacin. Third generation fluoroquinolones are active against both gram negative and gram positive bacteria including anaerobic bacteria. Furthermore, this generation is characterized by enhance potency, improved spectrum (includes anaerobes), and reduced resistance (Boothe, 2012). Pradofloxacin is distinguished from enrofloxacin, the first veterinary FQ, by two structure elements: a bicyclic amine, pyrrolidino-piperidine, replacing the ethyl-piperazine moiety located at position C-7 of enrofloxacin, and a cyano group which is attached to the C atom at position 8. FQs substituted at

position C-8 by a methoxy group were demonstrated to have greatly improved bactericidal activity, which was more pronounced for clones of *Escherichia* coli (Lu, 2001). Moreover, the mutant prevention concentrations (MPC) of such FQs were considerably lower than the MPCs of drug analogues carrying hydrogen at C-8 (Liu 2012). The most recent key modification was the observation that the addition of a methoxy group, instead of a halide, at the C-8 position specifically targets both topoisomerase II and IV, which also may decrease the possibility of the development of resistance to quinolones. Of the currently available agents, only gatifloxacin and moxifloxacin have a C-8 methoxy group in their chemical structure (Domgala, 1994; Zhao, 1997).

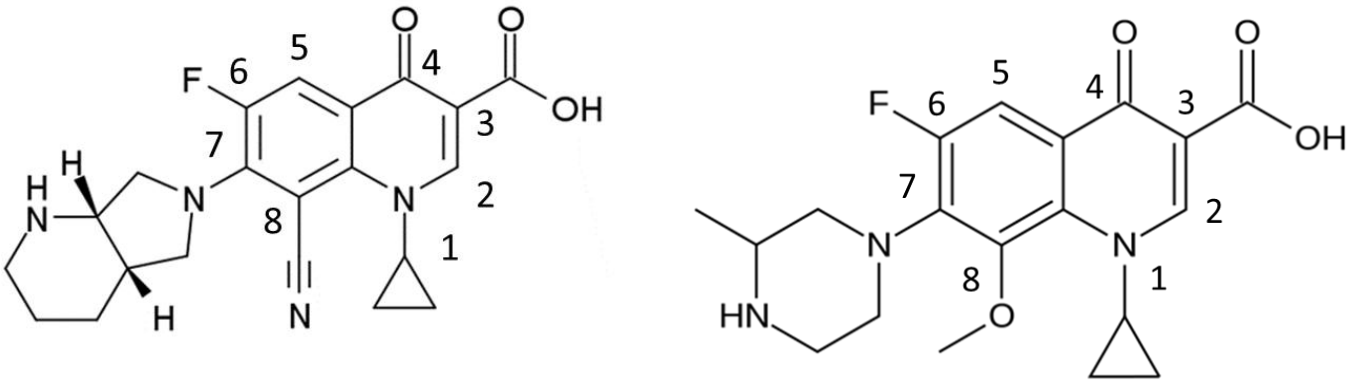


Figure 5. Structure of pradofloxacin (left) and gatifloxacin (right)

Mechanisms of Action

Appreciation of antimicrobial mechanisms of action allows for the identification of mechanisms by which antimicrobial resistance might be avoided or minimized.In Gram-negative bacteria, such as *E.coli*, fluoroquinolones principally inhibit DNA gyrase, whereas for Gram-positive organisms like *Staph. aureus*, Topoisomerase IV was found to be the principle target. By inhibiting these two target sites, this class of antibiotics is able to inhibit bacterial growth by blocking the DNA replication pathway (Alekshun, 2007). Fluoroquinolones are the only veterinary-approced antimicrobials that directly inhibit DNA synthesis (Boothe, 2012).

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 supercoils which must be removed to avoid stalling of the replication fork (Hopper, 1993). Overall, DNA gyrase moves ahead of the helicase and introduces nicks to the DNA strands and thus prevents helicase to stall or the DNA molecule to break.

During DNA synthesis, the double strands of the bacteria's circular DNA are in a tight, negative, coiled state. The DNA strands must then be uncoiled to allow DNA synthesis. Uncoiling the DNA strands induces stress and positive supercoiling, that must be removed. DNA gyrase is responsible for directing double-stranded breaks in the DNA and thus induces a negative supercoil, balancing positive supercoiling.. Therefore, DNA gyrase is an enzyme responsible for relieving stress while the double-stranded DNA is being unwound by helicase during DNA replication. Fluoroquinolones inhibit DNA synthesis through stabilizing the breaks in the DNA made by the DNA gyrase.

DNA gyrase is not present in mammals and thus presents as an excellent target to selectively inhibit bacterial growth. DNA gyrase has two subunits: *gyrA* and *gyrB*. The *gyrA* proteins contain the DNA-binding functions and they are also responsible for the induction of superhelical turns. Two fluoroquinolone molecules will bind to *gyrA* and prevent supercoiling. The nicks in the strand are then left exposed and this induces synthesis of repair enzymes called exonucleases which result in the breakdown of the DNA leading to the death of thebacterium (Hopper, 1993).

On the other hand, topoisomerase IV is a tetrameric enzyme in charge of unlinking DNA following DNA replication. Topoisomerase IV relaxes positive supercoils and decatenates DNA following DNA replication allowing the two daughter chromosomes to separate. Topoisomerase IV is comprised of two subunits: *ParC/GrlA* and *ParE/GrlB*. *ParC/GrlA* contains the DNA binding functions and therefore it is the target for fluoroquinolones. With topoisomerase IV, the rate at which replication is inhibited is 50 to 100 times slower than with DNA gyrase (Khodursky, 2004). The difference is generally explained by topoisomerase IV functioning behind replication forks, while DNA gyrase works ahead of them. Also, the enzymes differ such that DNA gyrase wraps DNA around itself, while topoisomerase IV does not. Moreover, DNA gyrase and topoisomerase IV can remove positive supercoils, but only DNA gyrase can convert them directly to negative supercoils. *gyrA* allows DNA gyrase to have a strong decatenating activity, much like that of topoisomerase IV (Kampranis, 2003). Furthermore, for the *E. coli* enzymes, inhibition of the decatenating activity of topoisomerase IV generally requires 15 to 50 higher fluoroquinolone concentration than inhibition of the supercoiling activity (Kato, 2000).

Molecular Mechanisms of Fluoroquinolone Resistance in *E.coli*

A substantive increase in fluoroquinolone resistance in companion animal *Escherichia coli* (*E.coli*) isolates has been reported (Shaheen et al., 2010) including that associated with multi-drug resistance (MDR). Moreover, fluoroquinolones are among the most common antimicrobials to treat urinary tract infection (UTI). Therefore, the prevalence of fluoroquinolone resistance can be linked to the increased use of broad spectrum antimicrobials at veterinary hospitals (Cooke et.al. 2002; Cohn et.al,; 2003; Boothe et.al., 2006).

First Step: Mutations in Target Sites

Mutations present in antibiotic target sites are often seen present in antibiotic resistant bacteria. Fluoroquinolones are known to target DNA gyrase and topoisomerase IV. Therefore, resistance to fluoroquinolones often emerges at low-levels by acquisition of initial resistance-conferring mutations in the enzymes DNA gyrase and topoisomerase IV (Piddock, 1999). Shaheen et.,al. (2011) also states that fluoroquinolone resistance in E coli appeared to be a stepwise phenomenon, with MIC increasing as the number of point mutations in *gyrA* increased, followed by mutations in *parC* and overexpression of AcrAB efflux pump.

Depending on the type of bacterium, these enzymes represent either the primary or secondary target of antimicrobial action. In *E.coli*, point mutations involved in fluoroquinolone resistance have been shown to occur in defined regions in the *gyrA* and *gyrB* genes, termed

quinolone resistance-determining regions (QRDRs), and those in the *parC* and *parE* genes of topoisomerase IV have been reported to occur in similar regions (Piddock, 1999). Mutations in *gyrB* and *parE* genes are less prevalent and rarely contribute to quinolone resistance (Giraud et al., 2001) Double mutations in *gyrA* gene are generally required for high-level resistance (Conrad et al., 1996) whereas mutations in *parC* are less frequent and are associated with lower level resistance (Bagel et.al., 1999; Everett et al., 1996) In gram negative bacteria, mutations in DNA gyrase occur first, whereas in gram positive, mutations in topoisomerase IV arise initially in a stepwise movement (Alekshun, 2007). Although mutations in *gyrA* and *parC* are a common cause of fluoroquinolone resistance they are not necessarily related to cause MDR.

(1) Mutations in DNA gyrase

Amino acid changes in the quinolone-resistant-determining region (QRDR) of *gyrA* alter the structure of the site of quinolone binding near the interface of the enzyme and DNA. This change in conformation leads to reduced drug affinity for the modified enzyme DNA complex. The level of resistance to fluoroquinolones is strongly correlated to codon specificity and is affected by the number of mutations present. For instance, a single mutation in *gyrA* in *E.coli* was identified to lead to high-level resistance to nalidixic acid (Markham and Neyfakh, 1996). Nonetheless, further *gyrA* mutations (and/or the topoisomerase IV) mutations play an essential role in the emergence of high-level resistance to fluorquinolones (Everett et.al., 1996; Ozeki et.al., 1997). Ozeki (1997) and White (2000) have demonstrated that in *E.coli* the mutation that occurs at codon 83 involves the substitution of serine residue for leucine, tryptophan or alanine.

A further mutation at codon 87 involving the substitution of aspartate generates slightly higher resistance to fluoroquinolones. Codon 87 involves the single substitution of aspartate for glycine, asparagine and tyrosine (Saenz et. al., 2003). Yoshida (1990) states that mutations at codons Ser83 and Asp87 confer much higher level of resistance than mutations in any other codon. Hooper (1999) states that each of these mutations prevents the drug from binding to the target site, rendering the antibiotic ineffective.

(1) Mutations in topoisomerase IV

Topoisomerase IV plays a secondary role as a target for quinolones in E.coli (Hoshino et.al., 1994). *parC* and *parE* of *E.coli* are the major sites for mutations to occur within topoisomerase IV. These mutations seem to arise once mutations in *gyrA* have occurred. DNA gyrase mutations should proceed topoisomerase IV in order to acquire high-level resistance. Therefore, stepwise mutations in *gyrA* and *parC* result in an increased incidence to develop resistance to fluoroquinolones (Shaheen, 2001). Overall, FQ resistance in *E. coli* occurs in a stepwise fashion and generally is associated with multidrug resistance (MDR), leading to therapeutic failure (Ruiz, 2003). Liu (2012) was able to demonstrate a clear relationship between the increasing MICs associated with the number of mutations in target genes by illustrating that the number of mutations in DNA gyrase and topoisomerase IV could be correlated with the level of MICs in a stepwise manner.

Second Step: Overexpression of Efflux Pump Activity

The cell wall of *E.coli* consists of both an inner and outer membrane separated by a periplasmic space. Located in the outer membranes of *E.coli*, efflux pumps are responsible for actively extruding foreign substances -including antimicrobials- and thus decrease intracellular concentrations of the antimicrobial

In addition to topoisomerase IV and DNA gyrase mutations, further second-step mutations associated within regulatory factors that control efflux pump expression and usually show a 2-8 fold increase in quinolone resistance levels (Jellen, 2001). Mutations conferring high-level resistance are often a mixture of both target- and efflux-related mutations with the latter mutations enhancing the expression of efflux pump activity (Liu et.al, 2012). Therefore, multi drug resistant isolates often confer second step mutations.

Genotype	MIC ($\mu g \cdot mL^{-1}$)
Wild-type	0.015[a]
GyrA substitution at Ser83	2–4[b]
GyrA substitution at Ser83 + Asp87	4–8[b]
ParC substitution + 1 GyrA substitution	4–8[b]
ParC substitution + 2 GyrA substitutions	8–32[b]
Down regulation of OmpF	0.25–0.5[c]
Over expression of AcrAB-TolC	0.12[a]
Over-expression of MarA	0.12[a]

Table 1. Effect of resistance mechanisms on MIC of ciprofloxacin (Piddock, 2001)

Table.1 further illustrates how the first step in fluoroquinolone resistance involves point mutations in target site *gyrA*, followed by mutations in *parC*. The presence of point mutations in the target sites allows for an increase in the MIC of ciprofloxacin of up to 32-fold. Second step

mutations are attributed to overexpression of efflux pumps, in particular AcrAB-TolC. If overexpression of AcrAB-TolC effluc pump occurs, there is usually a 2-8 fold increase in quinolone resistance. Over expression of efflux pumps results in a multi-drug phenotype (MDR) and clinical resistance (Piddock, 2006).

Efflux pumps

It is assumed that the evolutionary role of the MDR efflux pump is to protect the bacteria against a hostile environment. Consequently, in the act of protecting themselves against exogenous toxins, overexpression of these pumps allows further development of resistance to antimicrobials.

Furthermore, efflux pumps are responsible for intrinsic resistance to antibiotics since they are able to actively extrude antibiotics back into the environment. By actively extruding the antibiotic, the bacterium are able to survive in the presence of noxious agents. Efflux pumps are of interest due to their possible contribution to clinical resistance, possible targets (when inhibited), and their potential value in cell based screening for novel antibacterials (Sulavik et al., 2001).

MDR efflux pumps can be classified into 5 distinct families of proteins present in both Gram-negative and Gram-positive bacteria: (1) Resistance Nodulation Division (RND) family, which are the main pumps in Gram negative bacteria; (2) Major Facilitator Superfamily (MFS) which is expressed only in gram positive bacteria; (3)Staphylococcal Multi-Resistance (SMR), which is most commonly found in gram positive bacteria but some are expressed in gram negative bacteria; (4) Multi-Drug and Toxic Compound Extrusion (MATE) which are Na^+

proton pumps in gram negative bacteria only; and (5) ATP Binding Cassette (ABC), the only family that utilizes ATP hydrolysis as its energy source (Huguet, 2013).

AcrAB Efflux Pump

Because it is the major efflux pump of *E.*coli, this study focused on AcrAB. which belongs to the RND family. RND efflux pumps are organized as a tripartite efflux pump, meaning that it has three components. AcrAB is composed of a transporter protein in the inner membrane (AcrB), a periplasmic accessory protein (AcrA) and an outer membrane protein channel (TolC) (Koronakis et. al., 2004). AcrB captures its substrates within the inner membrane of the cytoplasm and effluxes them out into the external medium via TolC (Aires and Nikaido et.al., 2005). AcrA is the protein responsible for connecting AcrB and TolC. Moreover, the AcrAB efflux pump is a proton antiporter, meaning that it uses a proton gradient to exchange one H+ for one drug molecule (Paulsen, 2003).

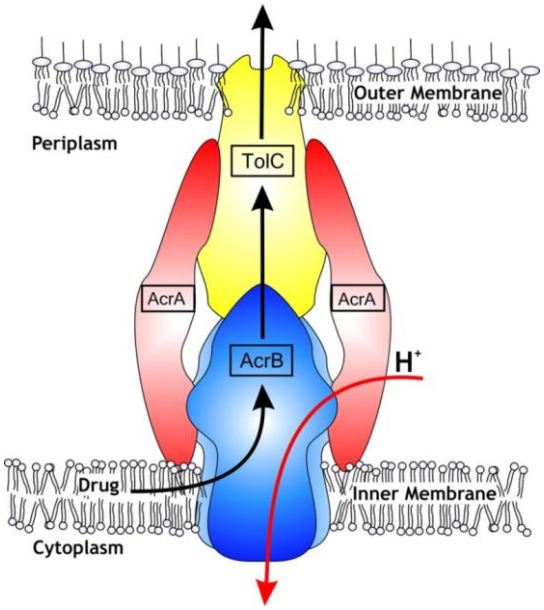


Figure 6. Schematic illustration of AcrAB-TolC (Blair, 2009).

The AcrAB-TolC efflux pump system has a wide substrate range including quinolones, tetracyclines, chloromphenicol, ampicillin, rifampicin, desinfectants and detergents (Breines, 2007).

Although other classes of pumps are expressed in *E.coli*, AcrAB-TolC has been found to be overexpressed by clinical isolates (Mazzariol, 2001). The AcrAB MDR efflux pump overexpression is associated with high-level FQ resistance in *E. coli* (Webber and Piddock 2001; Wang et al. 2003; Liu et al. 2012). However, overexpression of the AcrAB efflux pump alone does not confer clinical levels of resistance. Rather, as has been demonstrated for ciprofloxacin, overexpression of the AcrAB efflux pump must be accompanied by first-step mutations in a topoisomerase gene (Webber, 2001).

Mazzariol (2001) concluded in his studies that ciprofloxacin resistant isolates from humans and animals showed a 90% and 31% overexpression, respectively, of both *acrA* and *acrB.* Moreover, Oethinger (2000) states that AcrAB efflux pump is critical to fluoroquinolone resistance since deletion of AcrAB efflux pump, removes the ability to actively efflux ciprofloxacin. Moreover, *E.coli* cells bearing the acrAB deletion accumulated ciprofloxacin to more than twice the level seen for acrAB-positive cells. Although cells bearing acrAB deletion became much more susceptible to ciprofloxacin, mutants with newly acquired mutations in gyrA

still retained some gyrA-mediated fluoroquinolone resistance. However, this was well below the level of clinical significance.

Transcriptional Activation of AcrAB Efflux Pump

AcrAB efflux pump expression is regulated by upstream factors belonging to the AraC/XylS family of transcriptional activators named *soxS* and *marA* (Martin et al., 2002). Mutations that affect such regulatory genes also leads to fluoroquinolone resistance since the latter genes are responsible for regulating the intracellular drug concentrations by producing increased efflux of the drug (Oethinger, 2000).

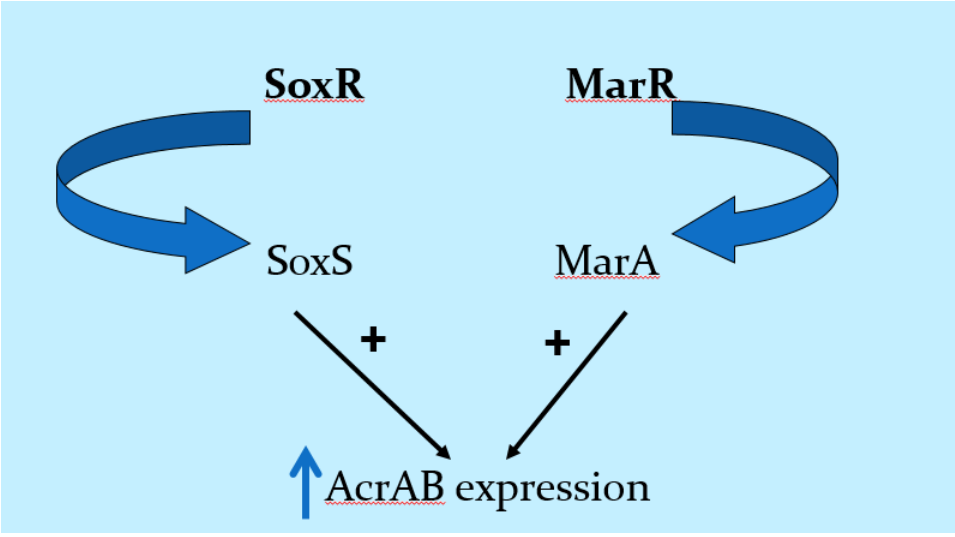


Figure 7. Illustration of AraC/XylS transcriptional activators and their role in AcrAB efflux pump expression.

soxS is regulated by the transcriptional activator SoxR while *marA* is regulated by MarR. Therefore, AcrAB pump is regulated by SoxS and MarA and these in turn are regulated by soxR and marR respectively. Constitutive expression of *soxS* has been reported to occur in response to mutations acquired within the C terminus of SoxR (Nunoshiba and Demple, 1994). Similarly, *marA* expression is depressed by mutations in *marR* (Oethinger et.al, 1998). AcrAB is normally produced at low levels but becomes de-repressed when under stress (Ma D, 1995). De-repression of acrAB does not lead to high level fluoroquinolone resistance but rather a low level increase (2-4 fold) in the MIC for most antibiotics (Breines, 1997).

soxRS Activation: A stress response

Hydrogen peroxide, superoxide and hydroxyl radicals are reactive oxygen species (ROS) formed as byproducts of respiration and they are classified as Their interactions with proteins and nucleic acids leada to cell stasis and death (Imlay, 2008). However, organisms have multiple protective pathways that scavenge ROS and prevent or repair the damage caused by ROS formation. When the concentration of ROS overcomes the ability of the cell to prevent its formation or repair the damage left behind, the outcome is oxidative stress. The soxRS system is one of the main protective responses to ROS exposure in *E.coli* .Moreover, the *soxRS* system

encodes two separate transcription activators, *soxR* and *soxS*, that participate in a two-step activation cascade (Krapp, 2011).

Transcriptional activators increase the transcription of a gene or set of genes and also ensure that the correct genes are transcribed at the correct amounts and the correct time. SoxR is a dimeric transcriptional activator belonging to the MerR family. SoxR is produced constitutively and is composed of two monomer clusters, each containing a 2Fe-2S group. When *E.coli* is exposed to redox-cycling drugs or superoxide radicals, the clusters undergo a reversible one-electron oxidation [Fe^{3+} - Fe^{3+}]. Upon oxidation, SoxR becomes a powerful transcription factor that gains the ability to bind to the site between the -10 and -35 elements of the *soxS*

promoter and activate the *de novo* transcription of *soxS*, the gene immediately adjacent (Figure 9) (Pomposiello, 2001). SoxS is also the only known target of the activated SoxR (Shah, 2004).

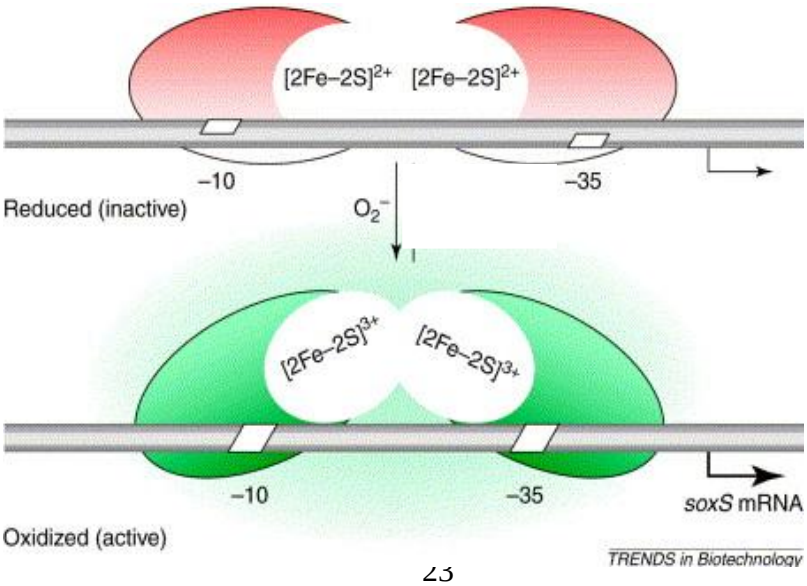


Figure 8. Mechanism of SoxR activation by superoxide (Pomposiello, 2001).

When oxidative stress hauls, the oxidized SoxR is returned to its reduced state via reducing systems and proteolysis rapidly degrades the extant SoxS protein, ending the response (Griffith *et al*., 2004). In the absence of ROS, SoxR is reduced and inactive. However, it still is able to bind to the *soxS* promoter but cannot enhance the transcription of *soxS*.

Hidalgo, 1997 states that transcription of soxS is very low in the absence of oxidized SoxR. Moreover, a series of 1-2 base pair engineered deletions in the *soxS* promoter dramatically increases Sox-R independent expression of *soxS* and although SoxR binds to the promoter tightly, the promoters are no longer activated by SoxR. The fact that reduced SoxR is still able to bind to the promoter but lacks transcriptional activity, indicates that transcriptional activation has to involve structural changes in the DNA-protein complex (Pomposiello, 2001).

Transcriptional Activator *soxS*

Once oxidized, SoxR binds to the *soxS* promoter to allow for the transcriptional activation of *soxS*. SoxS is synthesized *de novo* as response to oxidative stress and it is responsible of activating an array of genes that will combat the stress present. Thus, SoxR is the sensor of oxidative stress and SoxS is the response regulator (Shah, 2004).

The activity of *soxS* is controlled by its intracellular concentration and once transcribed, SoxS binds to a "marbox" or "soxbo." binding sites. Marboxes are located upstream of all the genes SoxS regulates. Moreover, SoxS binds to the marbox and interacts with RNA polymerase to activate the transcription of the genes of interest (Eaves et.al,2004).

Several features differentiate SoxS from other bacterial transcription activators. First, it is synthesized *de novo* in response to oxidative stress despite the rapidity with which ROS can potentially cause lethal lesions. Second, it is a very small molecule composed of 107 amino acid residues and it functions at a monomer. Moreover, SoxS is rapidly assembled (about 7 seconds per monomer at a polypeptide chain elongation rate of 15 amino acid residues per second) when compared with other transcriptional factors (Bremmer, 1996). The rapidity in assembling SoxS most likely compensates the fact that it is synthesized *de novo* at the onset of oxidative stress.

The genes SoxS transcriptionally activates include *sodA* (manganese-containing superoxide dismutase), *zwf* (glucose-6-phosphate dehydrogenase), *fldA* and *fldB* (two distinct flavodoxins), *fpr* (NADPH-ferredoxin reductase), *fur* (another gene regulator that is mainly involved with iron metabolism), *nfo* (DNA repair endonuclease IV), *acrAB* (efflux pump) and *micF* (an untranslated small RNA that down regulates the expression of the porin OmpF) (Storz, 1999) (Figure.10)

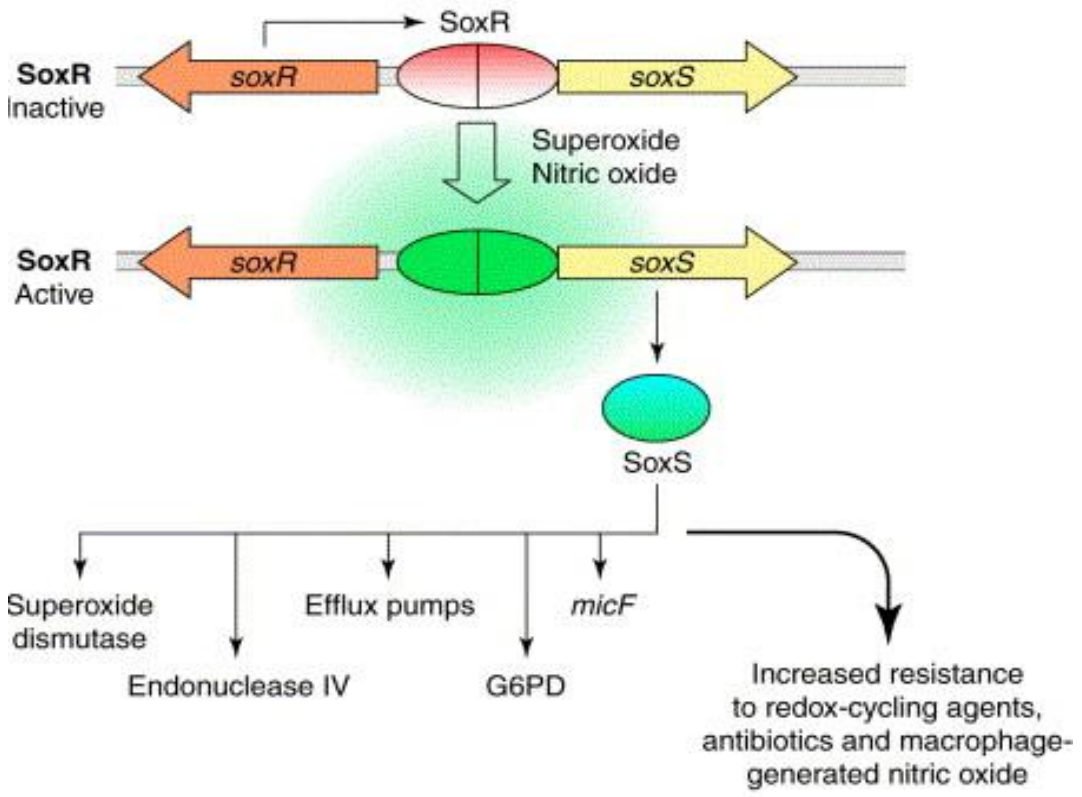


Figure 9. The SoxRS regulon and the genes SoxS transcriptionally activates (Pomposiello, 2001)

E.coli respond to an array of environmental challenges by coordinating and controlling the expression of certain genes. Ma (1996) states that transcription of acrAB was up regulated in strains of *E.coli* constitutively producing SoxS. This data correlates with Greenberg,'s(**2001)** observation that activation of the *soxRS* target genes by *E.coli* allows it to become resistant to not only ROS but also to antibiotics. Moreover, Amabile-Cuevas (1991) states that the *soxRS* response system is transcriptionally activated by reactive oxygen species to increase resistance to antibiotics and other agents via the AcrAB-TolC efflux pump.

AcrAB efflux pump expression activated by SoxS

Previous studies have attributed AcrAB overexpression in E.coli isolates to mutations in transcriptional regulators: *marR* and *soxR*. Ma (1995) demonstrated that mutations in global regulator *marR* was associated with increased transcription of AcrAB. Nunoshiba and Demple (1994) have demonstrated constitutive expression of *soxS* in response to mutations in the C terminus of SoxR. Constitutive expression of soxS in *E.coli* has also been associated with increased acrAB expression (Oethinger, 1998). High-level resistant *E.coli* clinical isolates constitutively express the *marA* or *soxS* genes (Maneewannakul, 1996).Therefore, Miller (1996) has stated that both MarA and SoxS confer resistance to antibiotics by activating or depressing a number of regulatory genes.

Purpose of Study

Previous work has been done in the Clinical Pharmacology Lab related to AcrAB efflux pump activity and its transcriptional regulators. Liu (2012) studied the relative expression of acrB and soxS in NDR, SDR and MDR E.coli isolates and observed that the expression of acrB increased with the severity of the resistance phenotype. Therefore, acrB was most highly expressed in MDR isolates. On the other hand, Debavayla (2009) identified a novel mutation in *soxS* in canine fecal *E.coli* isolates after dogs were treated with therapeutic doses of enrofloxacin but not amoxicillin. These isolates each expressed MDR. Ali (2012) described the mutation as a single mis-sense mutation in *soxS* pertainting to 3 of the MDR *E.coli* isolates from Debavyla's

study. This mutation was found in codon 12 with a G→T transversion leading to a substitution of alanine for serine. This or other mutations were not identified in any of the SDR or wild-type E.coli isolates. Although much research and importance has been given to the relationship between AcrAB efflux pump and fluoroquinolone resistance, Vissser (2011) has observed that after 30 minutes of ciprofloxacin exposure, EmrE efflux pump (belonging to SMR family) activity was overexpressed and much higher than the expression of AcrAB efflux pump.

Moreover, Liu (2013) compared the in vitro potency of newer versus earlier FQs among differing resistant phenotypes of companion animal *E.coli* isolates in order to assess whether or not later generation FQs offer a clinical advantage for treatment. The studies descriptors included measures of potency (MIC andMPC) as well as in vitro efficacy (based on the ratio of MIC/MICBP-S, MIC/MICBP-R, and MSW). The study suggests that pradofloxacin has the \ lowest MPC recorded in the study; mean MPC was two to five times lower than that of enrofloxacin. The study further demonstrated that FQ with the C-8 methoxy (such as pradofloxacin) compared to those with no substitution are characterized by lower MPC (Ince and Hooper 2001; Kowalski et al. 2003; Wetzstein 2005). The conclusion of the author was that a higher generation drug might be more prudent due to the poor performance of enrofloxacin; it was characterized by the least potency, and was second only to ciprofloxacin in terms of magnitude of MSW

Overall, previous studies in our lab have attempted to characterize mechanisms that E.coli develop in order to render antimicrobials ineffective. Liu (2013) has further attempted to characterize potency differences in FQs pertaining to different generations in order to describe

whether later generation FQs offer clinical advantage for treatment. Therefore, this study is a follow up and a complement to Liu's (2013) and Ali's (2012) study.

The purpose of this study was to evaluate the impact of exposure of different generations of fluoroquinolones on the mutability of *soxS* in clinical canine *E.coli* isolates associated with spontaneous disease. The drugs studied were ciprofloxacin, marbofloxacin (second generation fluoroquinolones),pradofloxacin and gatifloxacin (third generation fluoroquinolones). The study considered the impact of these drugs among differing resistance phenotypes of *E.coli* (ATCC, SDR, NDR and MDR) and examined the relationship of mutations and exposure to increasing concentrations (0.06-4µg/m, L) and exposure time (30, 60, 90 and 120 minutes) for each drug. The fluoroquinolone concentrations chosen represented 2-67 X the isolates respective fluoroquinolone MIC. In order to investigate the possible impact of any mutation on efflux pump activity, this study also measured relative expression of AcrAB and EmrE efflux pumps in isolates with or without mutations after fluoroquinolone exposure. This study will contribute to the accumulated knowledge regarding mechanisms whereby fluoroquinolones cause MDR, thus providing evidence for selecting one antimicrobial over another.

CHAPTER II
Fluoroquinolone-induced mutations:
Screening *soxS*

Introduction

The AcrAB MDR efflux pump overexpression has been shown to be associated with high-level fluoroquinolone (FQ) resistance in *E. coli* (Webber and Piddock 2001; Wang et al. 2003; Liu et al. 2012). AcrAB efflux pump expression is regulated by upstream factors belonging to the AraC/XylS family of transcriptional activators such as *soxS* (Martin et al., 2002).SoxS regulates intracellular drug concentrations by producing increased efflux of the drug (Oethinger, 2000). Boothe et al (2012) has identified a novel mutation in *soxS* (A12S) and these mutants are accompanied by overexpression of AcrAB and thus multi-drug resistance.

Newer generations of fluoroquinolones have not only increased in spectrum and potency but also appear to have decreased the incidence of resistance (Ball, 2000). For instance, third generation FQs (i.e, pradofloxacin) are substituted at position C-8 by a methoxy group and have demonstrated to have greatly improved bactericidal activity, which was more pronounced for clones of *Escherichia coli* (Lu, 2001).The most recent key modification was the addition of a methoxy group, instead of a halide, at the C-8 position specifically targets both topoisomerase II and IV, which also may decrease the possibility of the development of resistance to quinolones.. Gatifloxacin and moxifloxacin are examples of currently approved fluoroquinolones that have a C-8 methoxy group in their chemical structure (Domgala, 1994; Zhao, 1997).

In this study, broth macrodilution was performed in order to expose clinical canine *E.coli* isolates to increasing two-fold dilutions (2-64 X FQ MIC) of ciprofloxacin, marbofloxacin (second generation FQ), pradofloxacin and gatifloxacin (third generation FQ) for 30, 60, 90 and 120 minutes .The purpose of this study was to demonstrate, *in vitro,* whether different generations of fluoquinolones can predictably induce mutations in *soxS* in clinical canine *E.*coli isolates and to determine the impact of differing exposure times and concentrations to the emergence of mutations upon exposure to these drugs..

Materials and Methods

***E. coli* strains** –

Isolates (n=3) for each phenotype were randomly selected from a working subpopulation of isolates. This working subpopulation had been selected to represent, based on MIC distribution and resistance phenotypes, a study population of 3000. This surveillance population was acquired between May 2008 and June 2010. Isolates had been cultured from canine or feline urine samples of animals suspected of UTIs and submitted to veterinary diagnostic laboratory (IDEXX Reference Laboratories, Inc.) for identification and confirmation. Upon receipt in our laboratory, each *E. coli* isolate was re-cultured on BBL CHROMagar® *E.coli* agar plates (CHROMagar, Paris, France) at 37°C for 18-24 h to confirm isolate identification as *E. coli*. Isolates were stored at -80°C in trypticase soy broth/glycerol cyrovials (30%) until testing after susceptibility testing to 6 drugs classes (15 drugs): ampicillin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, meropenem, enrofloxacin, gentamicin, doxycycline, chloromphenicol, cefotaxime/clavulanic acid and

trimethoprim/sulfamethoxazole). Six drug classes were represented by these drugs, including the β-lactams (penicillins and cephalosporoins), tetracyclines, chloramphenicol, fluoroquinolones, aminoglycosides and folic acid inhibitors. The canine isolates for this study (n= 3) were categorized into the following resistance phenotypes: no drug resistance (NDR; n = 1), resistant to a single drug or drug class (SDR, resistance was expressed only to beta-lactams; n = 1) or resistant to two or more classes of antibacterial agents, i.e., multiple (MDR; n = 1).

Fluoroquinolone MIC of *E.coli* strains-

The MIC (µg/mL) for ciprofloxacin and enrofloxacin for each strain were respectively, NDR 0.03 for both drugs, 0.06 and 0.03 for SDR and 32 for both drugs in the MDR isolate.

Antimicrobial Exposure-

Using a checkerboard approach and broth macrodilution *E.coli* isolates were exposed to two-fold concentrations ranging from 4-0.06 µg/mL of ciprofloxacin, marbofloxacin, gatifloxacin and pradofloxacin (SigmaAldrich ®) .for 30, 60, 90 and 120 minutes. These concentrations represent 2-64 times the MIC of the respective exposed isolates (Table 3).

	4 µg/mL	2µg/mL	1µg/mL	0.5µg/mL	0.25µg/mL	0.12µg/mL	0.06µg/mL	0 µg/mL
30 mins								
60 mins								
90 mins								
120 mins								

Table 2. Checkerboard methodology illustrating antimicrobial concentrations and exposure times

A stock antimicrobial solution for ciprofloxacin, marbofloxacin, pradofloxacin and gatifloxacin was prepared at a concentration of 1mg/ mL by diluting all four antimicrobial agents in double distilled water solution (CLSI M31-A3). An intermediate working solution was prepared from the stock solution to obtain a final concentration of 8 µg/mL. This concentration was selected since it is two-fold higher than the desired starting antimicrobial concentration of 4 µg/mL.

Inoculum Preparation-

Start cultures of *E. coli* SDR, NDR, MDR, and ATCC reference strain 25922 (American Tissue Cell Culture, Manassas, Virginia, USA) were grown on Trypticase Soy Agar plates (Difco, MD) and incubated at 37°C for 18-24 hours. Each one of the three colonies representative of each *E.coli* phenotype, including ATCC, were then selected and transferred to 5mL of 0.9% saline solution. Samples were then adjusted to 0.5 McFarland standard turbidity (10^{-8} CFU). For the intermediate organism solution, the volume of standardized suspension to be added to Muller-Hinton Broth (MHB) was calculated to obtain a final organism concentration of approximately 1.5 X 10^{6} CFU/mL. The suspension was used within one hour of preparation.

Broth Macrodilution Checkerboard:

MHB was used to prepare six serial two fold dilutions established for each fluoroquinolone. For each isolate, 1mL of the prepared inoculum was dispensed to each tube with the resulting suspension = 7.5 X 10^5 CFU/mL The dilution tubes were then capped and stored in a shaker at 37 Cº and 250 rpm. The positive control contained media and inoculate but no fluoroquinolone and the negative control only contained media.

Selection of samples:

At each elapsed time point (0, 30,60, 90 and 120 mins), using a 10µL inoculating loop, drug-free Trypricase Soy Agar (TSA) plates were inoculated by subculturing from each of the dilution tubes. The TSA plates were then stored and incubated at 37ºC for 20 hours. 3 colonies were selected from each plate where growth was present. Samples that were collected using a 10µL inoculating loop were categorized as preliminary samples.

After the initial studies revealed limited *soxS* mutations, a second set of studies were implemented, with a minor modification such that a greater number of colonies might be recovered post exposure. Following fluoroquinolone exposure, each dilution tube was centrifuged for 10 minutes and 100µL from the bottom of each tube were pipetted on to drug-free TSA plates. By altering the methodology used to collect the preliminary set of samples, the study was able to increase the bacterial population that was to grow in drug-free TSA plates. 3 colonies were selected from each plate where growth was present.

Therefore, two different sample collection methods were utilized: an inoculating loop and 100µL from the bottom of each dilution tube.. In total, 6 colonies were collected from each plate where growth was present and these 6 colonies were further divided into two groups depending on the collection method utilized.

***soxS:* Gene Amplification**

Prior to (baseline) and after incubation, bacterial DNA extraction was performed in plates for which visible growth was present. Three separate *E.coli* colonies were selected from each plate where there was growth present and DNA was extracted from these individual colonies. Using an inoculating loop to select the colonies, each colony was suspended in 100µL of PreMan® Ultra Sample Preparation Reagent (Applied Biosystem, Foster City, CA) at 95ºC for 15 minutes, followed by centrifugation. DNA purity was assessed using a Nanodrop 2000® spectrophotometer (Thermo Scientific, Wilmington, DE).

PCR for *soxS* was carried out in a total volume of 25µL containing 50 pmol of each primer, 10 X PCR buffer, dNTPs, 50 mM $MgCl_2$, of *Taq* polymerase and DNA template. An 864bp fragment was amplified using the following primers:

soxS-F: 5'-TTGTTGAAACGCTGACCAC-3'

soxS-R: 5'-CCAGCGGAATGCCAATA-3

The *soxS* gene is only 324 bp and it is embedded in the middle of this selected 864 bp sequence. The 864 bp sequence was selected so that none of the 324 bp sequence would be lost in purification or sequencing.

Amplification of *soxS* was performed on the LightCycler® 480 instrument (Roche Applied Science, Indianapolis, IN) in the conditions stated in Table 4.

Conditions	Temp	Time	cycle
Denaturation	94	3 mins	1
Annealing	94	30 sec	30
	53	30 sec	
	68	45 sec	
Final Extension	72	7 min	1

Table 3. PCR Cycling conditions for amplification of *soxS*

Once PCR was performed, PCR products were ran through a 1.5 % agarose gel to confirm and identify the presence of the 864 bp amplicon.

DNA sequencing

Once the amplification of soxS was confirmed, the samples were stored in -20ºC. PCR products were purified and sequenced by Macrogen, Inc. (Maryland, USA) using an ABI 3730XL Genetic Analyzer for gene sequencing (Applied Biosystems, Foster City, CA). Only the forward strand was sequenced.

The ABI 3730XL Genetic Analyzer uses the Sanger sequencing method to be able to sequence specific genes. The Sanger sequencing method has been used since the mid 1980's and this method is useful for targeting a specific DNA sequence by using specifically designed primers to amplify the selected template of interest. Once the primers have annealed to the gene of interest, ddNTPs (dideoxynucleotides) are added to the solution.

The Sanger sequencing method not only uses dNTPs but also ddNTPs (dideoxynucleotide). Dideoxynucleotides differ from dNTP's because they contain a hydrogen group on 3'carbon instead of a hydroxyl group (OH). When ddNTP's are integrated into a growing chain of DNA, they stall growth since they prevent the further addition of dNTP's, The reason why the chain stalls is because the modified ddNTP's prevent the formation of a new phosphodiester bond between ddNTP's and the incoming dNTP's (Hartl, 2002),

Therefore, the terminator base (ddNTP) is incorporated and terminates the growing DNA chain. Moreover, each of the four ddNTPs has a different color tag that corresponds to each of the four nucleotides. This results in chains of varying lengths ending with different colored tagged ddNTP's. These dyes all fluoresce at different wave lengths. These fragments are then electrophoresed to assign a specific position using the ABI 3730 XL gene sequencer. Using this machine, a laser is reads the gel to determine the identity of the band according to which wavelength it fluoresces (Hartl, 2002). The results are then depicted on a chromatogram that identifies both the nucleotide and the position in the sequence of interest.

Upon arrival, the 864 base pair DNA sequences obtained from Macrogen Inc (Maryland, USA) were copied to an online software called Multalin (Corpet, 1988). This program allows the

copy of multiple DNA sequences, aligns them accordingly and compares the sequences to each other and their respective controls. Using Multalin, the 324 base pair fragment pertaining to *soxS* of each sample was selected and screened for DNA point mutations.

Amino acid sequencing

Once the 324 base pair *soxS* sequence was selected from each of the isolates, the sequences were copied and pasted to ExPASy Translate Tool (SIB Swiss Institute of Bioinformatics). This online program is able to translate a specific DNA sequence to an amino acid sequence. The corresponding ORF (Open Reading Frame) was selected and the amino acid sequence for each sample was copied and pasted in Multalin once again. Multalin allows the copy of multiple amino acid sequences, aligns them accordingly and compares the sequences to each other and their respective controls.

Results

Pre-exposed *E.coli* isolates

ATCC ®25922, SDR, NDR and MDR were initially sequenced for s*oxS* prior to fluoroquinolone exposure. The DNA and amino acid sequences were aligned and compared to

the *soxS* sequence of *E.coli* K-12 obtained from GenBank. No mutations in *soxS* were identified in any of the pre-exposed isolates. Moreover, no amino acid mutations were identified in non-exposed isolates allowed to grow for 30, 60, 90 and 120 minutes.

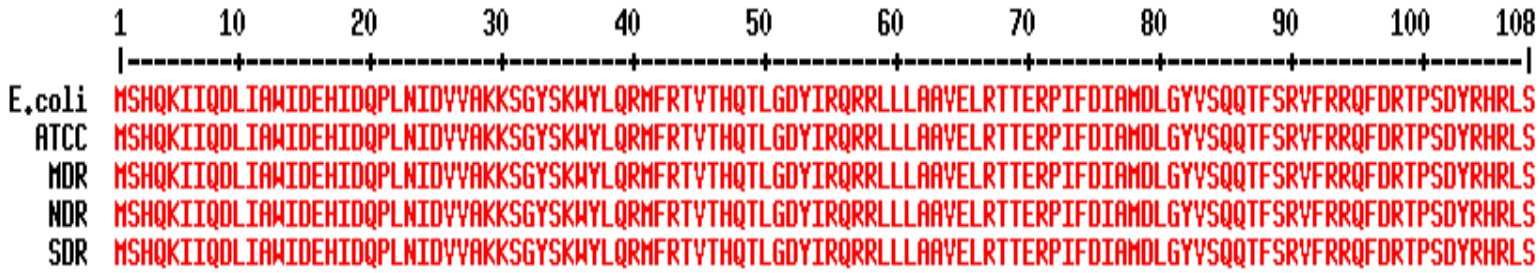


Figure 11. Alignment of *soxS* amino acid sequences of pre-exposure isolates

Marbofloxacin, Pradofloxacin and Gatifloxacin exposed *E.coli* isolates

Following *soxS* amplification and sequencing, no nucleotide or amino acid mutations were identified in any of the isolates exposed to marbofloxacin, pradofloxacin or gatifloxacin.

Ciprofloxacin exposed isolates

soxS mutations were only identified in ciprofloxacin exposed isolates. Following ciprofloxacin exposure and overnight incubation, visible growth was recorded for each of the exposed isolates. MDR exposed to ciprofloxacin was able to grow at all the allotted concentrations and time points. However, the same growth pattern was not observed for the

remaining three phenotypes (SDR, NDR and ATCC) which were not able to grow past 0.25µg/mL of ciprofloxacin and as exposure time increased, growth also decreased.

Identification of *soxS* DNA mutations

No mutations were identified in the *soxS* sequence of any post- ciprofloxacin exposed MDR isolates. However, *soxS* base pair mutations were identified in ATCC, SDR, and NDR ciprofloxacin exposed isolates.

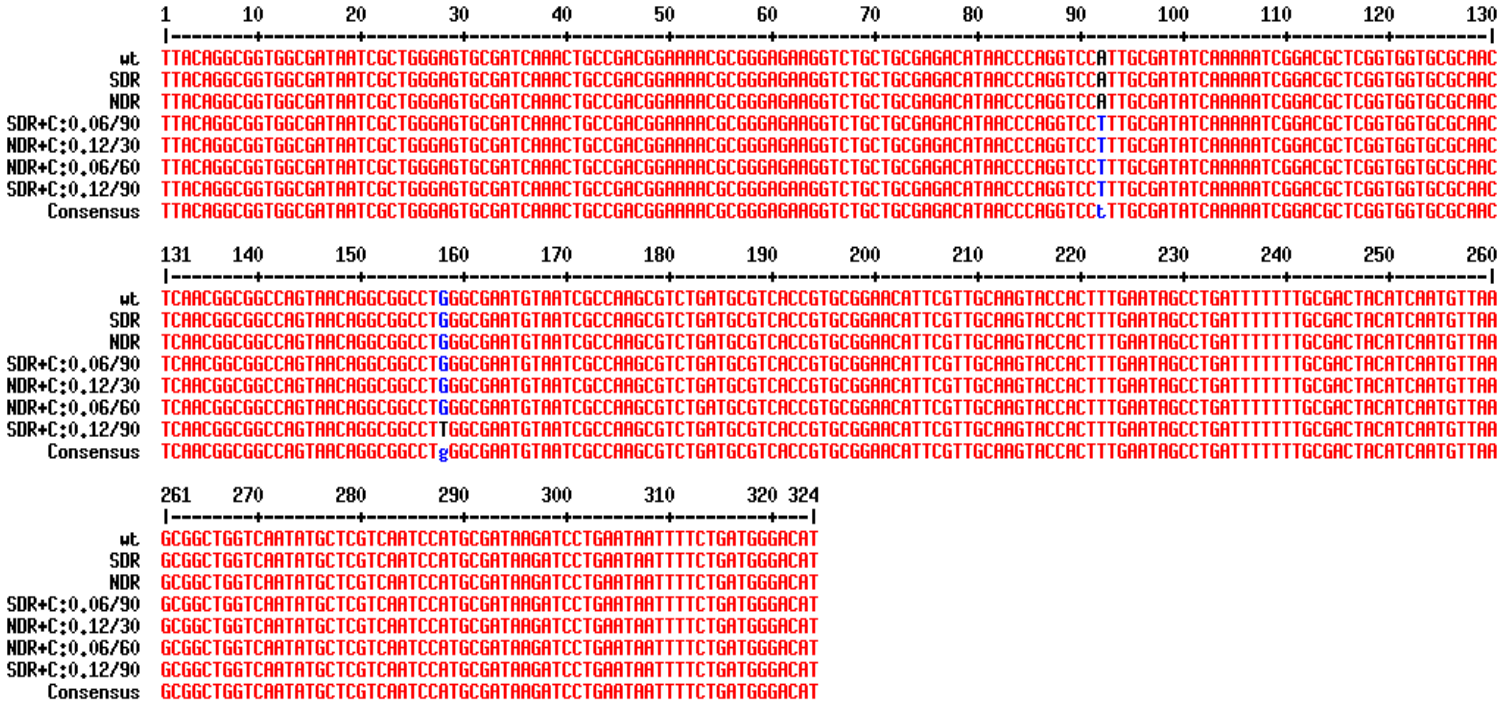
All point mutations identified were categorized as transversions, exchangine a purine for a pyrimidine base. Transervsions were identified after only 30 minutes of ciprofloxacin exposure. Also, the mutations identified occurred after exposure to 0.06µg/mL and 0.12µg/mL. Table 8 provides a summary of the point mutations identified in post- ciprofloxacin exposure *E.coli* isolates. Whereas each colony sampled using the inoculating loop carried the same mutation, only one of the three colonies collected by sampling 100µL had the mutations identified in Table 8.

Isolate Phenotype	Ciprofloxacin Exposure		*soxS* sequence
	Concentration (µg/mL)	Exposure time (minutes)	Nucleotide change
NDR	0.06	30	0
NDR	0.12	30	A→T
NDR	0.06	60	A→T
SDR	0.25	30	0
SDR	0.12	30	0
SDR	0.06	30	0
SDR	0.12	60	0
SDR	0.06	60	0
SDR	0.12	90	A→T
			G→T
SDR	0.06	90	A→T
ATCC 25922	0.25	30	0
ATCC 25922	0.06	30	G→T
			A→C
ATCC 25922	0.06	60	A→C

Table 7. Summary of point mutations identified in post-exposure *E.coli* clinical isolates

Table 4. Summary of point mutations identified in soxS of E.*coli* clinical isolates of varying phenotypes after 30, 60 and 90 minutes of ciprofloxacin exposure

(A)


wt
SDR
NDR
SDR+C:0.06/90
NDR+C:0.12/30
NDR+C:0.06/60
SDR+C:0.12/90
Consensus

(B)

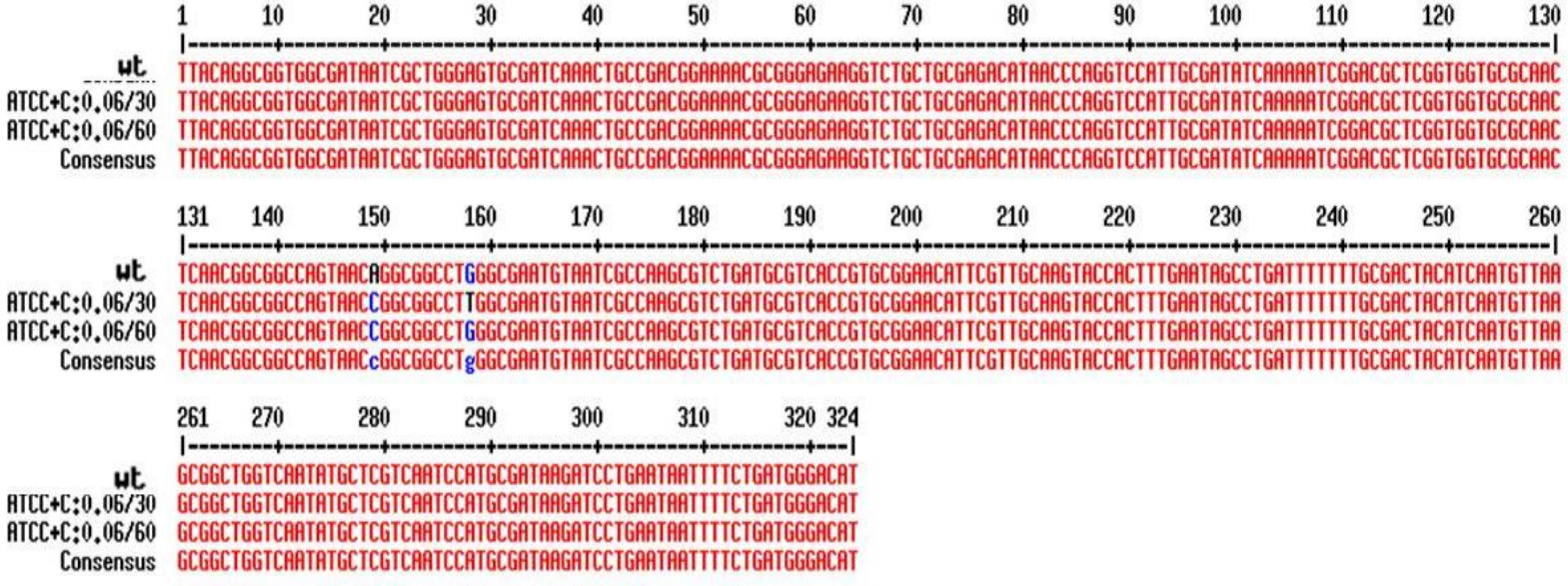

wt
ATCC+C:0.06/30
ATCC+C:0.06/60
Consensus

Figure 12. *soxS* DNA sequence alignment of *E.coli* K-12 (*wt)*, (A) SDR, NDR and (B) ATCC isolates exposed to 0.06 and 0.12µg/mL of ciprofloxacin for 30, 60 and 90 minutes

Identification of amino acid mutations

Point mutations and amino acid changes that occurred after such exchange are delineated in Figure 17. All identified amino acid mutations were determined to be mis-sense mutations, leading to a change in a single amino acid in the encoded protein.

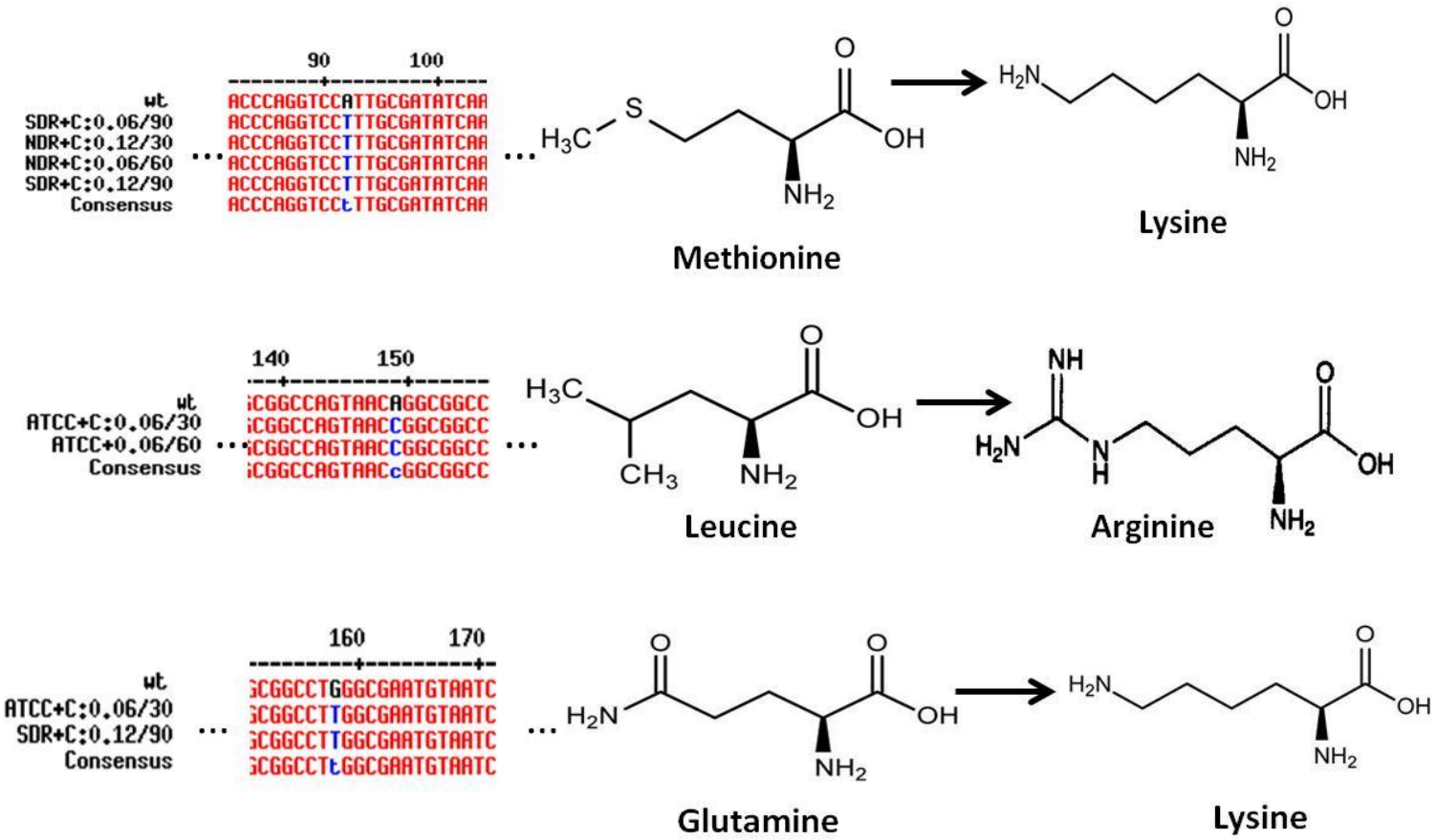


Figure 13. Summary of mis-sense mutations identified in *soxS* of ciprofloxacin exposed SDR, NDR, and ATCC

.

Three distinct mis-sense mutations were identified in ciprofloxacin exposed isolates. A transversion occurred in position 91, exchanging A→T. This point mutation lead to an amino acid change: methionine (M)→lysine (K). Another transversion occurred in position 148, exchanging A→C. This point mutation lead to an amino acid change: leucine (L)→arginine (R). Lastly, a transversion also occurred in position168, exchangine G→T. This point mutation lead to an amino acid change: glutamine (Q)→lysine (K) .

The same transversion (A→C) and amino acid change (L59R) was identified in the *soxS* sequence of ATCC exposed to 0.06µg/mLof ciprofloxacin for 30 and 60 minutes. ATCC exposed to 0.06µg/mL for 30 minutes and SDR exposed to 0.12µg/mL for 90 minutes shared the same transversion (G→T) exchanging Q56K. Lastly, SDR exposed to 0.12 and 0.06µ/mL of ciprofloxacin for 90 minutes and NDR exposed to 0.12 and 0.06µg/mL for 30 and 60 minutes, respectively, shared the same nucleotide change (A→T) leading to an amino acid exchange of M78k .Table 9 summarizes and identifies the point mutations that occurred, as well as the changes that occurred in mRNA codons.

Isolate Phenotype	Ciprofloxacin Exposure		*soxS* sequence	
	Concentration (µg/mL)	Exposure time (minutes)	mRNA codon change	Amino acid change
NDR	0.06	30	0	0
NDR	0.12	30	AUG→AAG	M78K
NDR	0.06	60	AUG→AAG	M78K
SDR	0.25	30	0	0
SDR	0.12	30	0	0
SDR	0.06	30	0	0
SDR	0.12	60	0	0
SDR	0.06	60	0	0
SDR	0.12	90	AUG→AAG	M78K
			CAG→AAG	Q56K
SDR	0.06	90	AUG→AAG	M78K
ATCC 25922	0.25	30	0	0
ATCC 25922	0.12	30	0	0
ATCC 25922	0.06	30	CAG→AAG	Q56K
			CUG→CGG	L59R
ATCC 25922	0.06	60	CUG→CGG	L59R

Table 5. Summary of *soxS* mis-sense mutations identified in *E.coli* isolates of various phenotypes exposed to 0.06 and 0.12µg/mL of ciprofloxacin for 30,60 and 90 minutes

Discussion

Previous studies have attributed efflux pump overexpression and thus antimicrobial resistance in *E.coli* isolates to mutations in transcriptional regulators such as *marR* and *soxR* (Ma,1995; Nunoshiba and Demple, 1994). However, Maneewannakul (1996) has stated that a proportion of high-level resistant *E.coli* clinical isolates have been shown to constituvely express *soxS* genes and Miller (1996) has stated that *soxS* confers resistance to antibiotics by activating or depressing a number of regulatory genes.

Ali (2012) was able to describe and identify a single mis-sense mutation in *soxS* codon 12 with a G→T transversion leading to a substitution of alanine for serine (A12S). Therefore, the goal of this study was to demonstrate, *in vitro,* whether exposure to ciprofloxacin, marbofloxacin, gatifloxacin or pradofloxacin can predictably induce the A12S or other mutations in *soxS.*

This study was not able to identify the previously identified mutation A12S demonstrated in fecal *E.coli* of dogs receiving enrofloxacin. However, exposure of SDR, NDR and ATCC to ciprofloxacin for as little as 30 minutes was able to induce novel mutations in the *soxS* gene. Moreover, *soxS* mutations were identified in *E.coli* isolates exposed to 2-4 times their established FQ MIC. Exposure to newer generation fluoroquinolones such as pradofloxacin, marbofloxacin and gatifloxacin did not induce any mutations in *soxS.*

All point mutations that were identified were categorized as transversions. The transversions that did occur also lead to mis-sense mutations where one single amino acid was changed in the encoded protein. Due to the possibility of the occurrence of spontaneous

mutations, all the *soxS* sequences pertaining to exposed isolates were compared to non-exposed isolates. Pre-exposed isolates had been allowed to grow for 30,60, 90 and 120 minutes in drug-free MHB. No mutations were identified in any of the *soxS* sequences pertaining to pre-exposed isolates. The fact that no mutations were identified in these samples, implies that the mutations encountered in post-exposed isolates did not occur due to spontaneous mutation. Moreover, increasing the number of colonies by collecting 100µL rather than just a 10 µL loop allowed the collection of a much higher concentration of bacteria and thus a truer representation of the population.

When comparing the incidence of mutation between NDR and SDR, the amino acid substitution occurred earlier in NDR. However, for SDR, after 90 minutes of ciprofloxacin exposure, two amino acid changes were identified. Moreover, no point mutations or amino acid substitutions were identified in MDR. MDR was the only isolate that was able to grow at all the allotted times and ciprofloxacin concentrations. Because the MDR isolate is resistant to fluoroquinolones, ciprofloxacin was not able to bind to its target sites and inhibit bacterial growth. Therefore, ciprofloxacin was not able to alter any molecular elements such as *soxS*.

The M78K substitution was identified in both SDR and NDR ciprofloxacin exposed isolates. Methionine (M) is a hydrophobic amino acid containing a large, non-reactive side chain that is ideally suited for packing in the protein interior. On the other hand, lysine (K) is an amphipathic amino acid generally found on the outside of proteins. Therefore, substituting methionine for lysine can potentially lead to structural changes in protein folding. Moreover,

lysines, unlike methionine, are quite frequent in protein active or binding sites (Betts, 2003). As a consequence, substituting methionine for lysine can have potential effects in protein activation.

The L59R substitution was identified in ATTC ciprofloxacin exposed isolates. Leucine (L) is a hydrophobic amino acid having an aliphatic side chain. Aliphatic side chains are non-reactive and are rarely directly involved in protein function. Moreover, since leucine is a hydrophobic amino acid, it prefers to be buried in protein hydrophobic cores. On the other hand, arginine (R) is an amphipathic amino acid, having hydrophobic and polar areas. Argininines are commonly found in the surface of the protein and are frequently identified in protein active or binding sites (Betts, 2003). Substituting leucine for arginine can lead to structural changes in protein folding due to the difference between the hydrophobic nature of leucine and amphipathic nature of arginine. Substituting leucine for arginine can also have potential effects in protein activity due to the substitution of leucine, a non-reactive amino acid, to arginine, an amino acid usually found in the active site of proteins.

The Q56K substitution was identified in both ATCC and SDR exposed isolates. Glutamine (Q) is an amphipathic amino acid and it is quite frequently involved in protein active or binding sites. Moreover, lysine (K) is also an amphipathic amino acid (Betts, 2003). Both of these amino acids share this similarity and thus both prefer to be on the outside of the protein, with the hydrophobic side chain buried within the protein. Due to their common amphiphatic nature, the Q56K substitution may not lead to disastrous effects in protein folding and structure.

It is clear that the positioning and properties of amino acids is key to understanding many biological processes. Therefore, by analyzing and comparing amino acid properties, structures

and functions, one is able to have an idea of the significance in substituting one amino acid for another. Even though understanding and comparing these characteristics provides insight for the impact of amino acid mutations, protein modeling would provide further understanding of the role these mutations play in protein structure .

Conclusion

It was the goal of this study to demonstrate, *in vitro*, whether ciprofloxacin, marbofloxacin, pradofloxacin and gatifloxacin can predictably induce previously identified mutations (A12S) or other mutations in *soxS.* This study was able identify three novel mutations (M78K Q56K and L59R) in the *soxS* gene when exposing SDR, NDR and ATCC isolates to 0.06 and 0.12µg/mL of ciprofloxacin for 30.60 and 90 minutes.

This study was able to demonstrate that ciprofloxacin, a second generation fluoroquinolone, was the only of the four studied fluoroquinolones that was able to induce a mutation in *soxS.*

Two of the identified mutations (M78K and L59R) were identified as being significant amino acid substitutions due to differences in structure and function between the amino acids involved in the mis-sense mutations. However, further studies are needed to evaluate the mis-sense mutations identified using protein modeling.

Chapter III

AcrB and EmrE efflux pump expression in ciprofloxacin exposed Isolates

Introduction

The cell wall of *E.coli* consists of both an inner and outer membrane separated by a periplasmic space. Located in the outer membranes of *E.coli*, efflux pumps are responsible for actively extruding foreign substances -including antimicrobials- and thus decrease intracellular concentrations of the antimicrobial**.** Furthermore, efflux pumps are responsible for intrinsic resistance to antibiotics since they are able to actively extrude antibiotics back into the environment. By actively extruding the antibiotic, bacteria are able to survive in the presence of noxious agents. Efflux pumps are of interest due to their possible contribution to clinical resistance, possible targets (when inhibited), and their potential value in cell based screening for novel antibacterials (Sulavik et al., 2001).

The AcrAB MDR efflux pump overexpression has been shown to be associated with high-level fluoroquinolone resistance in *E. coli* (Webber and Piddock 2001; Wang et al. 2003; Liu et al. 2012). Mazzariol (2000) concluded in his studies that ciprofloxacin resistant isolates from humans and animals showed a 90% and 31% overexpression, respectively, of both *acrA* and *acrB.* Moreover, *soxS* is a transcriptional activator of AcrAB and Aly (2012 Ali (2012) has described a single mis-sense mutation in *soxS* pertainting to 3 MDR *E.coli* isolates. These same isolates also showed overexpression of *acrB*. Furthermore, although much research and importance has been given to the relationship between AcrAB efflux pump and fluoroquinolone

resistance, Vissser (2011) has observed that after 30 minutes of ciprofloxacin exposure, EmrE efflux pump (belonging to SMR family) activity was overexpressed and much higher than the expression of AcrAB efflux pump.

In this study, different phenotypes of *E.coli* isolates (SDR, NDR, MDR and ATCC) were exposed to varying concentrations of fluoroquinolones (ciprofloxacin, marbofloxacin, pradofloxacin and gatifloxacin) for 30, 60, 90 and 120 minutes. Sequence analysis confirmed the presence of *soxS* mutations when SDR and NDR were exposed to 0.06 and 0.12µg/mL of ciprofloxacin. RNA was extracted from these isolates. Due to the fact that *soxS* mutations were only identified in ciprofloxacin exposed isolates and that *soxS* is a transcriptional activator of AcrAB efflux pump, it is the interest of this study to evaluate AcrB expression in clinical *E.coli* isolates in which the soxS mutation occoured and those in which it did not after exposure to ciprofloxacin. Furthermore, EmrE efflux pump expression was evaluated as a follow up to Visser's (2011) findings. We hypothesized that efflux pump expression would be higher in isolates with *soxS* mutations when compared to non mutants and non-fluoroquinolone exposed isolates.

Materials and Methods

E.coli samples

Broth macrodilution was performed to expose clinical *E.coli* SDR and NDR isolates to 0.06 and 0.12µg/mL of ciprofloxacin for 0, 30, 60 and 90 minutes Figure 18 summarizes and identifies with an "X" the time points and concentrations of the samples. Pre-exposed isolates served as controls.

	Ciprofloxacin exposure (mins)			
	0	30	60	90
SDR				
0	X	X	X	X
0.06		X	X	X*
0.12		X	X	X*
NDR				
0	X	X	X	
0.06		X	X*	
0.12		X*		

Figure 14. Summary of ciprofloxacin concentrations and time points to which SDR and NDR isolates were exposed.

"X" means overnight growth was present
*represents isolates were *soxS* mutations were identified

RNA extraction

The level of expression of efflux pumps can be measured by quantitative real-time reverse transcriptase PCR (*q*RT-PCR). For RNA extraction, three bacterial colonies representing each phenotype, concentration and time point were harvested and suspended in 5mL of 0.9% normal saline and standardized to 0.5 McFarland standard turbidity ($\sim 10^8$) using the SENSITITER® Nephelometer.. 0.25ml of the standardized solution was added to 2.5ml of LB broth (BBL, MD) and allowed to grow at 37°C in a shaking incubator with an RPM of 200 for 2 hours in order to achieve an OD_{600} of 0.7-0.8, corresponding to the mid-logarithmic phase. Following this growth, bacterial RNA extraction was performed using an RNeasy Mini Kit (Quiagen, Inc., Valencia, CA) following the protocol of the manufacturer. RNA concentrations and 260/280 were measured by using Nanodrop 2000® spectrophotometer (Thermo Scientific, Wilmington, DE). All 260/280 measurements were about 2.0, assuring RNA purity. The iScript™ cDNA Synthesase Kit (BioRad Laboratories, Inc., CA) was used to perform reverse transcriptase and thus obtain cDNA. Once again, DNA purity was assessed using the Nanodrop 2000®.

Relative expression of efflux pumps

Primers were first selected from genetic codes in GenBank and ordered from Eurofins MWG Operon® (Huntsville, AL). *gapA* was the housekeeping gene selected to perform *q*RT-PCR and the primer pairs used for the housekeeping gene were the following:

GapA-F- 5'TCCGACCCCAACGTATCTGTAG-3'

GapA-R-5'AACGCCTTTCATTTCGCCTTCA-3'

It is the interest of this study to measure both AcrB and EmrE efflux pump expression and thus the selected primers used to conduct *q* RT-PCR were the following:

AcrB-F-5'-AAACTGCCTACCGGTGTTGGCTAT -3'

AcrB-R-5'-TGAGCAGGCCTACCTGGAAGTAAA-3'

EmrE-F-5'- GCTCAGACGCTGGCTTATATTCCT-3'

EmrE-R-5'-ACCGGCACAAATCAACATCATGCC-3'

q RT-PCR was performed using the Roche ® Light-Cycler 480 and Roche ® DNA Master SYBR Green 1. Relative expression was chosen since it allows to compare levels and changes in expression of genes relative to a known reference gene with stable expression levels, in this case *gapA*. The relative expression level of both *acrB* and *emrE* was calculated by the level of relative quantification in the target gene divided by that of *gapA.* The expression of the target genes was defined as the relative expression level in each isolate divided by that of *E.coli* ATCC 25922. DNA from ATCC 25922 also served as the calibrator to perform *q* RT-PCR. Overexpression of the target genes was defined as > two-fold gene expression compared to that of ATCC 25922.

Statistical Analysis

Final expression levels for each concentration and time point were represented as the average of the three colonies representing each category. All the results were compared between concentrations and time-points by Holm-Sidak multiple comparisons (SigmaStat® and SigmaPlot® Version 12.2).

Results

AcrB expression

Expression of efflux pump activity significantly differed between time, concentration and isolates. *acrB* was identified to be overexpressed (2.2779) in NDR when exposed to 0.12 µg/mL for 30 mins (M78K). *soxS* mutation (M78K) was also identified when exposing NDR to 0.06 µg/mL for 60 mins. Although, *acrB* was not overexpressed in this isolate, a significant increase in *acrB* expression was identified when compared to the control (p=). Furthermore, no visible growth was present when exposing NDR to 0.12 µg/mL for 60 mins. (Figure.)

soxS mutations were identified when exposing SDR to 0.06µg/mL (M78K) and 0.12 µg/mL (M78K, Q56K) of ciprofloxacin for 90 minutes. Significant differences and increased a*crB* expression was identified between control and SDR exposed to 0.06µg/mL(p=) and 0.12µg/mL (p=) of ciprofloxacin for 90 minutes. Moreover, *acrB* was over expressed (2.141) in SDR when exposed to 0.12 µg/mL for 90 mins (M78K, Q56K) (Figure.)

Significant differences in *acrB* expression could not be detected between control and non-exposed SDR isolates allowed to grow for 30, 60 and 90 minutes (p=0.084). Also, significant differences could not be detected between control and non-exposed NDR isolates allowed to grow for 30 and 60 minutes (p=0.098).

.

	Exposure time (mins)							
Cipro concentration (µg/mL)	0		30		60		90	
NDR	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0.2345	0.0765	0.3014	0.0587	0.6754	0.0632		
0.06	-		0.5877	0.0589	1.9461*	0.0683		
0.12	-		2.2779*	0.2198	-			
SDR								
0	0.2876	0. 0654	0.3239	0.0724	0.4855	0.0722	0.5212	0.0573
0.06	-		1.0486	0.0257	1.3065	0.0541	1.4221*	0.0668
0.12	-		0.6775	0.0181	0.7172	0.0376	2.1411*	0.0852

Table 7. Summary of mean relative expression of *acrB* in pre and post ciprofloxacin exposed NDR and SDR

*means *soxS* mutation

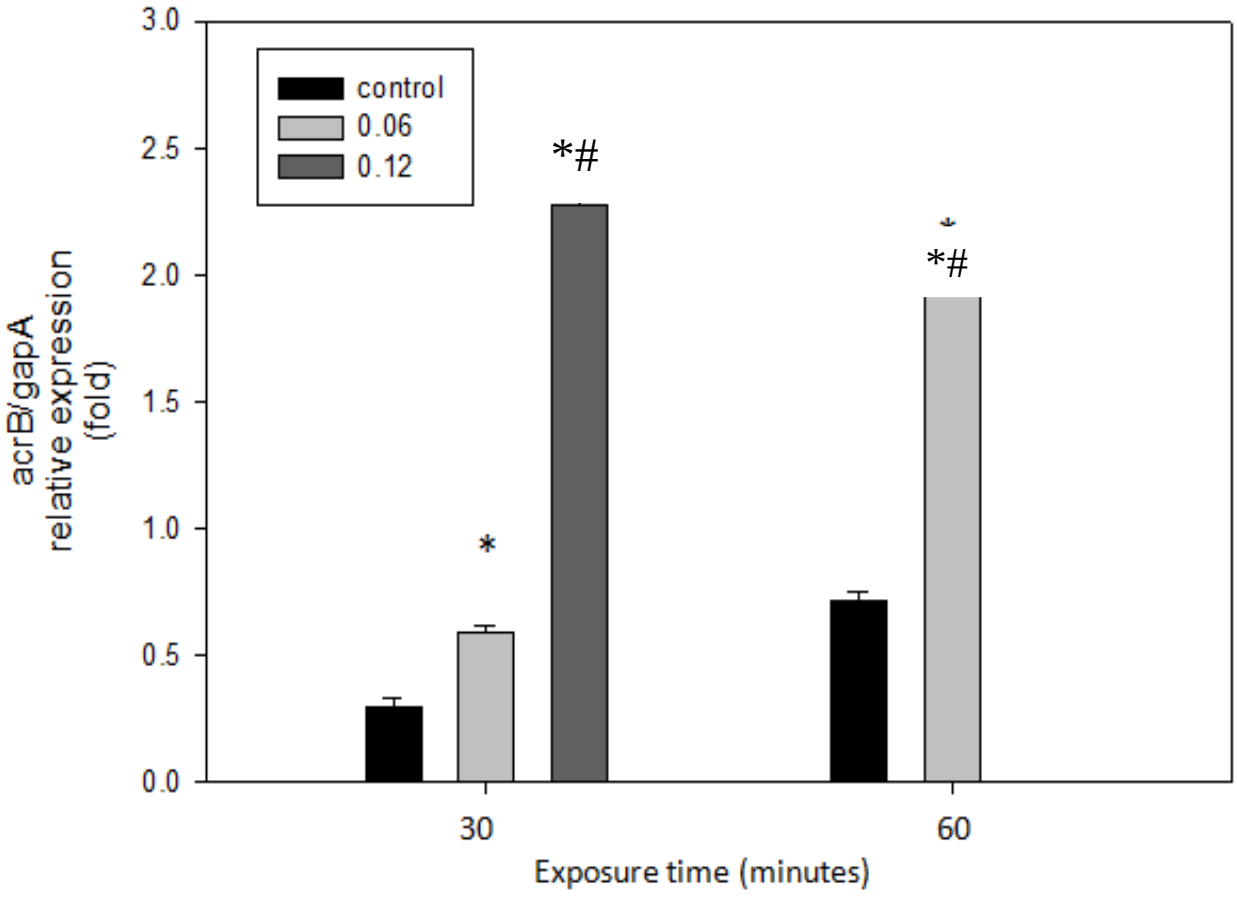


Figure. 15. *acrB/gapA* relative expression in non-exposed (control) NDR and after 30 and 60 minutes of 0.06 and 0.12ug/mL of ciprofloxacin exposure

#

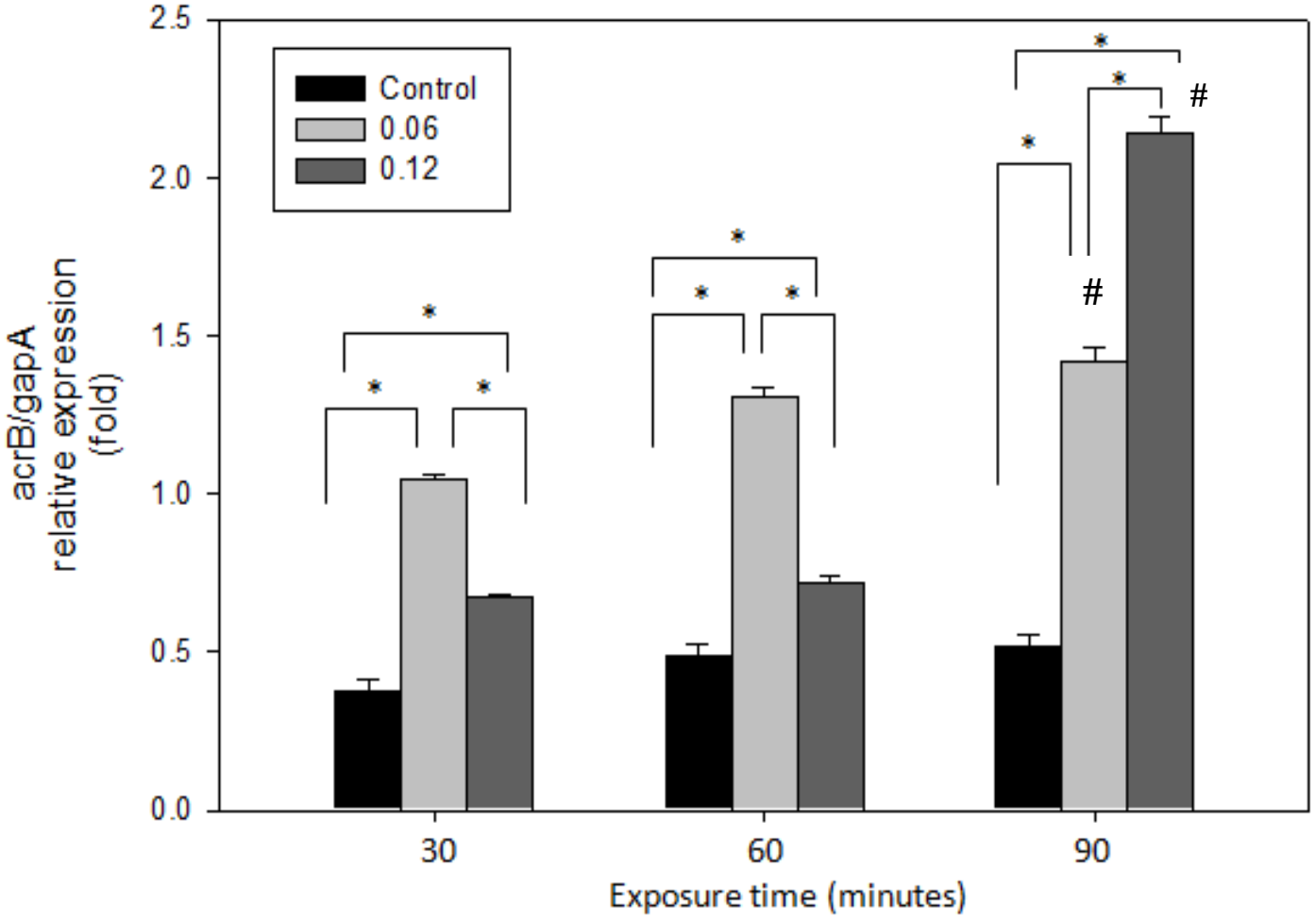


Figure. 16 *acrB/gapA* relative expression in non-exposed (control) SDR and after 30, 60 and 90 minutes of 0.06 and 0.12µg/mL of ciprofloxacin exposure
Note:* means P-value <0.05
means *soxS* mutation

EmrE expression

In NDR, at the 30 minute time point, *emrE* expression was greatest at 0.12 µg/mL(M78K) when compared to the control (p=0.017) and 0.06 µg/mL (p=0.025). The M78K *soxS* mutation was also identified when exposing NDR to 0.06µg/mL for 60 minutes. *emrE* expression was significantly greater in this isolate when compared to the control (p=0.05).

In SDR, at the 90 minute time point, *emrE* expression was greatest when exposed to 0.06 µg/mL of ciprofloxacin (M78K) when compared to control (p=0.017) and 0.12 µg/mL(M78K, Q56K) (p=0.050). *emrE* expression was significantly higher in the M78K, Q56K mutant when compared to the control (p=0.025).

Although overexpression of *emrE* was not detected, increased expression was identified in isolates bearing *soxS* mutations when compared to the control and non-mutants.

	Exposure time (mins)							
Cipro concentration (µg/mL)	0		30		60		90	
NDR	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0.2345		0.3456	0.0587	0.6753	0.0632		
0.06	-		0.5432	0.0551	1.2343*	0.1142		
0.12	-		1.7983*	0.1631	-	-		
SDR								
0	0.2212	0.0452	0.3758	0.0724	0.4568	0.0722	0.5623	0.0573
0.06	-		0.8331	0.1006	0.9484	0.0641	1.2639*	0.0684
0.12	-		0.6468	0.1561	0.7816	0.0432	0.9546*	0.0513

Table 7. Summary of mean relative expression of *emrE* in pre and post ciprofloxacin exposed NDR and SDR

*means *soxS* mutation

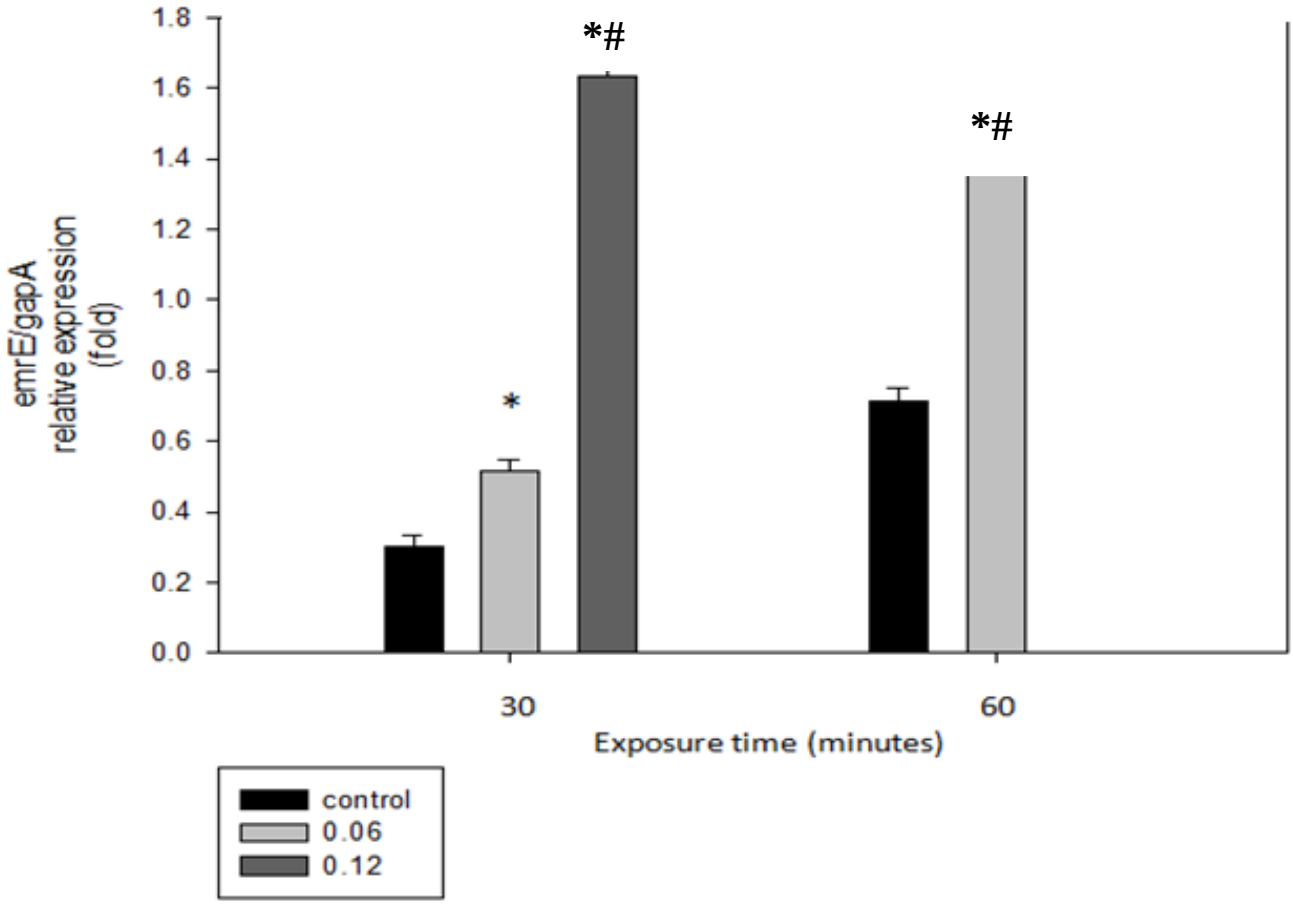


Figure. 17. *emrE/gapA* relative expression in non-exposed NDR and after 30 and 60 minutes of 0.06 and 0.12µg/mL of ciprofloxacin exposure

Note:* means P-value <0.05

identified *soxS* mutation

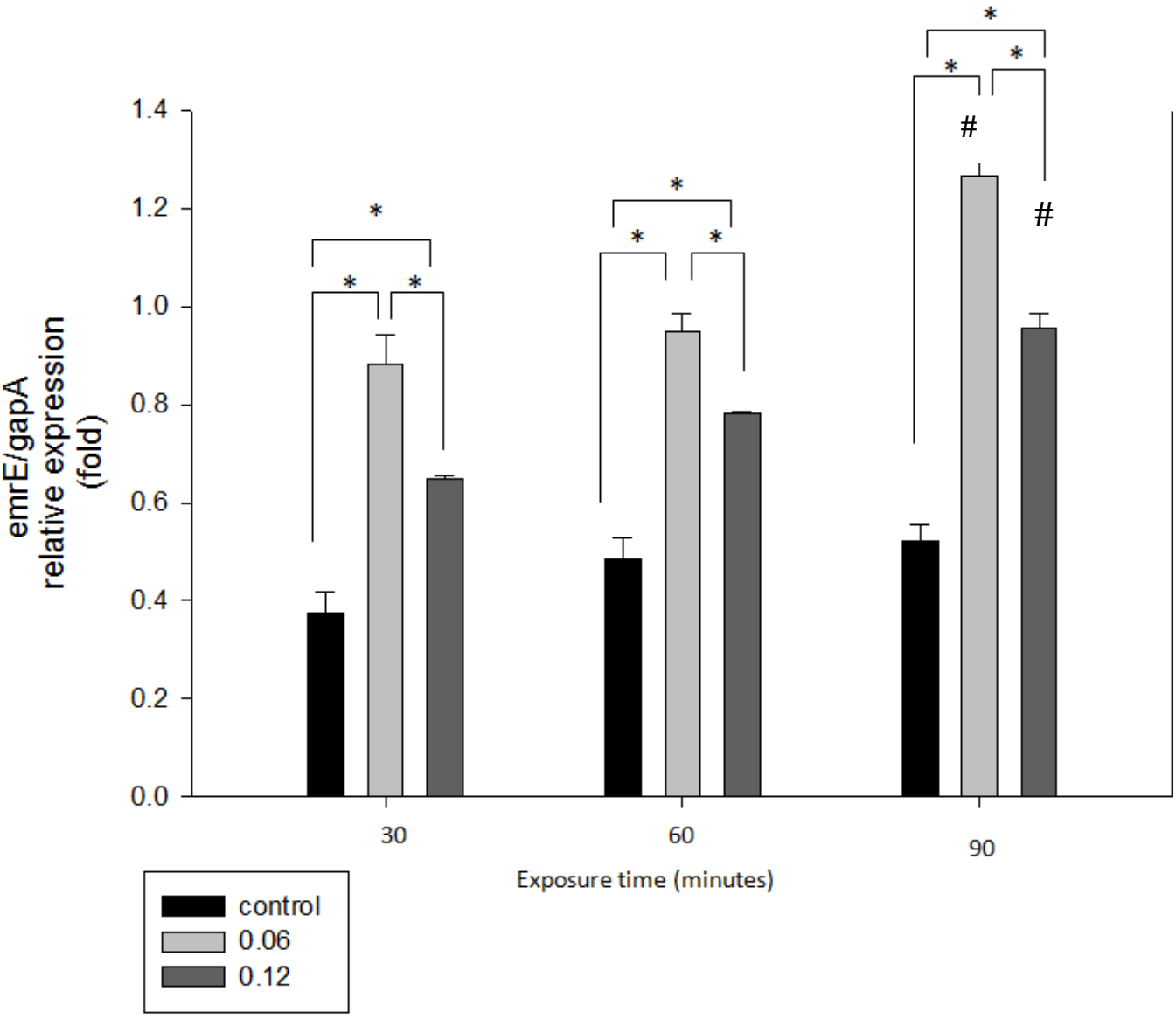


Figure 18. *emrE/gapA* relative expression in non-exposed SDR and after 30, 60 and 90 minutes of 0.06 and 0.12µg/mL of ciprofloxacin exposure

Note:* means P-value <0.05

identified *soxS* mutation

Discussion

This study was able to identify significant differences in efflux pump activity between non-exposed and ciprofloxacin exposed isolates (SDR and NDR) with and withough *soxS* mutations.

Overexpression of *acrb* (2.278) was identified when exposing NDR to 0.12µg/mL of ciprofloxacin for 30 minutes (M78K). The same *soxS* mutation was identified when exposing NDR to 0.06µg/mL for 60 minutes. Although overexpression of *acrB* was not identified in this isolate, expression was still significantly different and greater (+1.233) when compared to the control. However, higher exposure time precluded growth, indicating that, in the NDR isolate, increased pump expression could not overcome inhibitory effects of ciprofloxacin.

acrB was over expressed (2.141) in SDR when exposed to 0.12 µg/mL for 90 mins (M78, Q56K). The fact that ciprofloxacin exposed SDR was able to grow for a longer time frame when compared to NDR, might indicate that it has acquired resistance mechanisms that the NDR isolate lacks.

Increased and overexpression of *acrB* in M78K and Q56K *soxS* mutants might suggest that these mutations are significant enough to alter acrB efflux pump activity. The findings of this study identifying the relationship between *soxS* mutations and increased *acrb* expression

agree with Liu (2012) findings, in which he identified that expression of *acrB* significantly increased in the mutant when compared to a control.

Furthermore, Visser (2011) was able to identify over expression of EmrE efflux pump following 30 minutes of 10µg/mLof ciprofloxacin exposure. As a follow up, this study was interested in evaluating the relative expression of EmrE efflux pump after 30, 60 and 90 minutes of 0.06 and 0.12µg/mL of ciprofloxacin exposure. In contrast to Visser (2011) this study was not able to document overexpression of emrE after ciprofloxacin exposure. However, this study does document increased activity compared to control isolates. Like acrB, emrE expression increased in both NDR and SDR after exposure of 0.06 and 0.12 µg/ml ciprofloxacin for 30 minutes, despite a FQ MIC of 0.03-0.06 µg/ml. Moreover, emrE expression was greater at time points and concentrations were *soxS* mutations had been identified when compared to non-mutants. Increased expression of both *acrB* and *emrE* expression in ciprofloxacin exposed isolates might suggest that efflux pumps work synergistically in the presence of stress.

Future studies could measure post-fluoroquinolone MICs since conducting susceptibility testing on *soxS* mutant colonies would provide insight to whether the mutation impacts the MIC of the isolate. Furthermore, the use of efflux pump inhibitors could also be evaluated in order to provide better understanding on the role increased efflux pump expression has on the MIC of these samples.

Conclusions

Efflux pumps are a mechanism by which *Escherichia coli* develops clinical drug resistance. Resistance initially limited to fluoroquinolones (FQ) rapidly crosses to multiple drug classes, causing multidrug resistance. Previous research has indicated that an overexpression of AcrAB-TolC can be correlated with FQ resistance. Moreover, Visser (2011) has identified overexpression of EmrE in isolates exposed to ciprofloxacin.

This study indicates that a significant increase of both AcrB and EmrE occurred in clinical *E.coli* isolates (SDR and NDR) following exposure to ciprofloxacin. Moreover, overexpression of *acrB* was identified at concentrations and time points that had previously induced *soxS* mutations. Although over expression of *emrE* was not detected, a significant increase in expression was identified in ciprofloxacin exposed isolates. This should be further explored since EmrE efflux pump has not been associated with fluoroquinolone resistance.

Oethinger (2000) states that AcrAB efflux pump was found to be critical to the fluoroquinolone resistance level since upon the deletion of AcrAB efflux pump, the ability to actively efflux ciprofloxacin was completely lost. Moreover, research into quinolone derivatives as efflux pump inhibitors has indicated some promising results as well, and may well lead to the synergistic use of fluoroquinolones with other antimicrobial compounds (Mahamoud, et al., 2007). Our data suggests the rapid emergence of antimicrobial resistant mutants in clinical veterinary settings that can contribute to dissemination of drug-resistant commensal bacteria. Studies like this also allow possible explanations of the factors that affect efflux pump expression and thus aid elucidate molecular mechanisms that might lead to antimicrobial resistance.

Chapter IV:

General Conclusions

Escherichia coli is a major cause of urinary tract infections in dogs and cats and fluoroquinolones are among the drugs most commonly used to treat UTI. Nonetheless, a substantive increase in fluoroquinolone resistance in companion animal *E. coli* isolates has been reported (Shaheen et al., 2010) including that associated with multi-drug resistance (MDR). Because fluoroquinolones are used by veterinarians as first-line treatment in the United States and *E coli* is a common cause of urinary tract infection, its resistance is a medical concern since it leads to therapeutic failure in the veterinary patient. Moreover, emergence of antimicrobial resistance in *E.coli* isolates from small animal patients presents a concern not only in the realm of animal health, but it also presents health consequences to humans if these isolates are transmitted from their pets (Beutin *et. al*, 1999; Johnson *et.al*).

Among the mechanisms by which *E.coli* become resistant to fluoroquinoloneis decreased intracellular drug concentrationcaused by increased efflux pump activities. Among the different efflux transport proteins, the AcrAB-TolC system is the most active in *E coli*. AcrAB efflux pump overexpression has been shown to be associated with high-level fluoroquinolone resistance in *E. coli* (Webber and Piddock 2001) and Boothe et. al (2012) has described a single mis-sense mutation (A12S) in *soxS,* transcriptional activator of AcrAB, pertaining to 3 MDR *E.coli* isolates. These same isolates also showed overexpression of *acrB.* Moreover, Vissser (2011) has observed that after 30 minutes of ciprofloxacin exposure, EmrE efflux pump activity was overexpressed and much higher than the expression of AcrAB efflux pump. Therefore, the goal

of this study is to put these two findings together and demonstrate that *in vitro* exposure of isolates to concentrations of fluoroquinolones that are achieved clinically can induce *soxS* mutations and such mutations are associated with increased efflux pump expression.

Our study was not able to identify the previous identified mutation A12S. However, this study was able to identify three novel mutations (M78K Q56K and L59R) in *soxS* when exposing SDR, NDR and ATCC isolates to 0.06 and 0.12µg/mL of ciprofloxacin for 30,60 and 90 minutes. However, no point mutations were identified in MDR. It can be suggested that because the MDR isolate is resistant to fluoroquinolones, ciprofloxacin was not able to bind to its target sites and inhibit bacterial growth. Therefore, ciprofloxacin was not able to alter or interact with the replication pathway and thus did not alter any other molecular elements. Moreover, exposure to newer generation fluoroquinolones such as pradofloxacin, marbofloxacin and gatifloxacin did not induce any mutations in *soxS.* Therefore, this study was able to demonstrate, *in vitro,* that later generations of FQs were not able to predictably induce novel mutations in *soxS.*

Our findings also indicate that a significant increase in both AcrB and EmrE occurred in clinical *E.coli* isolates (SDR and NDR) following *in vitro* exposure to ciprofloxacin. Moreover, overexpression of *acrB* was identified at concentrations and time points that had previously induced *soxS* mutations. Although over expression of *emrE* was not detected in ciprofloxacin exposed isolates, a significant increase in expression was identified as exposure time increased. This should be further explored since EmrE efflux pump has not been associated with fluoroquinolone resistance.

Finally, this study has provided potential value in cell based screening for novel fluoroquinolones by addressing molecular mechanisms involved in antimicrobial resistance. This study has been able to address the relationship between *E.coli* efflux pump expression and *in vitro* ciprofloxacin exposure. By addressing this relationship, this study elucidates the important role efflux pumps play in antimicrobial exposure. The findings of this study are also able to describe the potential for emergence of antimicrobial resistance patterns by screening transcriptional activator *soxS* and by evaluating efflux pump expression in ciprofloxacin exposed isolates. Describing the impact fluoroquinolone exposure has on *soxS* and efflux pumps supports the possibility of these becoming potential targets for novel fluoroquinolones.

References

Mahamoud A, Chevalier J, Alibert-Franco S, V. Kern W, Page J. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. Journal of Antimicrobial Chemotherapy (2007) 59, 1223–1229

Fàbrega A, Martin RG, Rosner JL, Tavio MM, Vila J.Constitutive SoxS expression in a fluoroquinolone-resistant strain with a truncated SoxR protein and identification of a new member of the marA-soxS-rob regulon, mdtG. Antimicrob Agents and Chemotherapy. 2010 Mar;54(3):1218-25.

Drlica K, Schmitz FJ. Therapeutic options in an era of decreasing antimicrobial susceptibility. Journal of Chemotherapy. 2002 Feb;14 Suppl 2:5-12.

Drlica K, Zhao X. Mutant selection window hypothesis updated. Clinical Infectious Diseases. 2007 Mar 1;44(5):681-8.

Drlica K. The mutant selection window and antimicrobial resistance. Journal of Antimicrobial Chemotherapy. 2003 Jul;52(1):11-7

Boothe DM, Boeckh A, Simpson RB, Dubose K. Comparison of pharmacodynamic and pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. Journal of Veterinary Internal Medicine. 2006 Nov-Dec;20(6):1297-306.

Shaheen BW, Boothe DM, Oyarzabal OA, Smaha T. Antimicrobial resistance profiles and clonal relatedness of canine and feline Escherichia coli pathogens expressing multidrug resistance in the United States. Journal of Veterinary Internal Medicine. 2010 Mar-Apr;24(2):323-30

Beutin L. Escherichia coli as a pathogen in dogs and cats. Veterinary Research. 1999 Mar Jun;30(2-3):285-98.

Guardabassi L, Schwarz S, Lloyd DH.Pet animals as reservoirs of antimicrobial-resistant bacteria. Journal of Antimicrobial Chemotherapy. 2004 Aug;54(2):321-32.

Webber M, Piddock LJ.Quinolone resistance in Escherichia coli. Veterinary Research. 2001 May-Aug;32(3-4):275-84.

Webber MA, Piddock LJ.Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. Antimicrob Agents Chemother. 2001 May;45(5):1550-2.

Oluoch AO, Kim CH, Weisiger RM, Koo HY, Siegel AM, Campbell KL, Burke TJ, McKiernan BC, Kakoma I.Nonenteric Escherichia coli isolates from dogs: 674 cases (1990-1998). J Am Vet Med Assoc. 2001 Feb 1;218(3):381-4.

Andriole VT. The quinolones: past, present, and future. Clin Infect Dis. 2005 Jul 15;41 Suppl 2:S113-9.

Lu T, Zhao X, Li X, Drlica-Wagner A, Wang JY, Domagala J, Drlica K.Enhancement of fluoroquinolone activity by C-8 halogen and methoxy moieties: action against a gyrase resistance mutant of Mycobacterium smegmatis and a gyrase-topoisomerase IV double mutant of Staphylococcus aureus. Antimicrob Agents Chemother. 2001 Oct;45(10):2703-9.

Ball P.Safety of the new fluoroquinolones compared with ciprofloxacin. J Chemother. 2000 Jan;12 Suppl 1:8-11.

Domagala JM. Structure-activity and structure-side-effect relationships for the quinolone antibacterials. J Antimicrob Chemother. 1994 Apr;33(4):685-706.

Zhao, X., C. Xu, J. Domagala, and K. Drlica. 1997. DNA topoisomerase targets of the fluoroquinolones: a strategy for avoiding bacterial resistance. Proc. Natl. Acad. Sci. USA 94:13991-13996

Alekshun MN, Levy SB.Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007 Mar 23;128(6):1037-50.

Hoopper D, Ince D. Quinolone Resistance Due to Reduced Target Enzyme Expression. J. Bacteriol. Dec 2003 185 (23): 6883-6892

Cooke CL, Singer RS, Jang SS, Hirsh DC. Enrofloxacin resistance in Escherichia coli isolated from dogs with urinary tract infections.J Am Vet Med Assoc. 2002 Jan 15;220(2):190-2.

Cohn LA, Gary AT, Fales WH, Madsen RW. Trends in fluoroquinolone resistance of bacteria isolated from canine urinary tracts. J Vet Diagn Invest. 2003 Jul;15(4):338-43.

Piddock LJ.Mechanisms of fluoroquinolone resistance: an update 1994-1998. Drugs. 1999;58 Suppl 2:11-8.

Shaheen BW, Boothe DM, Oyarzabal OA, Wang C, Johnson CM.Evaluation of the contribution of gyrA mutation and efflux pumps to fluoroquinolone and multidrug resistance in pathogenic Escherichia coli isolates from dogs and cats. Am J Vet Res. 2011 Jan;72(1):25-32.

Markham PN, Neyfakh AA. Inhibition of the multidrug transporter NorA prevents emergence of norfloxacin resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 1996 Nov;40(11):2673-4.

Ozeki S, Deguchi T, Yasuda M, Nakano M, Kawamura T, Nishino Y, Kawada Y. Development of a rapid assay for detecting gyrA mutations in Escherichia coli and determination of incidence of gyrA mutations in clinical strains isolated from patients with complicated urinary tract infections. J Clin Microbiol. 1997 Sep;35(9):2315-9.

Yoshida H, Bogaki M, Nakamura M, Nakamura S. Quinolone resistance-determining region in the DNA gyrase gyrA gene of Escherichia coli. Antimicrob Agents Chemother. 1990 June; 34(6): 1271–1272.

Sáenz Y, Zarazaga M, Briñas L, Ruiz-Larrea F, Torres C. Mutations in gyrA and parC genes in nalidixic acid-resistant Escherichia coli strains from food products, humans and animals. Journal of Antimicrobial Chemotherapy (2003) 51, 1001–1005.

Hoshino K, Kitamura A, Morrissey I, Sato K, Kato J, Ikeda H.Comparison of inhibition of Escherichia coli topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob Agents Chemother. 1994 Nov;38(11):2623-7.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection.J Antimicrob Chemother 51, 1109–1117.

Jellen-Ritter AS, Kern WV.Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in Escherichia coli mutants Selected with a fluoroquinolone. Antimicrob Agents Chemother. 2001 May;45(5):1467-72.

Liu X, Boothe DM, Thungrat K, Aly S. Mechanisms accounting for fluoroquinolone multidrug resistance Escherichia coli isolated from companion animals. Vet Microbiol. 2012 Dec 28;161(1-2):159-68

Piddock LJ. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol. 2006 Aug;4(8):629-36.

Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N, Greene J, DiDomenico B, Shaw KJ, Miller GH, Hare R, Shimer G.Antibiotic susceptibility profiles of Escherichia coli strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother. 2001 Apr;45(4):1126-36

Koronakis V, Eswaran J, Hughes C.Structure and function of TolC: the bacterial exit duct for proteins and drugs. Annu Rev Biochem. 2004;73:467-89.

Aires JR, Nikaido H. Aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter of Escherichia coli. J Bacteriol. 2005 Mar; 187(6):1923-9.

Mazzariol A, Tokue Y, Kanegawa TM, Cornaglia G, Nikaido H.High-level fluoroquinolone-resistant clinical isolates of Escherichia coli overproduce multidrug efflux protein AcrA. Antimicrob Agents Chemother. 2000 Dec;44(12):3441-3.

Webber MA, Piddock LJ. Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. Antimicrob Agents Chemother. 2001 May;45(5):1550-2.

Oethinger M, Kern WV, Jellen-Ritter AS, McMurry LM, Levy SB.Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in Escherichia coli in the absence of the AcrAB efflux pump. Antimicrob Agents Chemother. 2000 Jan;44(1):10-3.

Martin RG, Rosner JL.Genomics of the marA/soxS/rob regulon of Escherichia coli: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol Microbiol. 2002 Jun;44(6):1611-24.

Imlay JA.Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem. 2008;77:755-76

Krapp AR, Humbert MV, Carrillo N. The soxRS response of Escherichia coli can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content. Microbiology. 2011 Apr;157(Pt 4):957-65.

Pomposiello PJ, Demple B.Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends Biotechnol. 2001 Mar;19(3):109-14.

Shah IM, Wolf RE Jr.Novel protein--protein interaction between Escherichia coli SoxS and the DNA binding determinant of the RNA polymerase alpha subunit: SoxS functions as a co-sigma factor and redeploys RNA polymerase from UP-element-containing promoters to SoxS-dependent promoters during oxidative stress. J Mol Biol. 2004 Oct 22;343(3):513-32.

Griffith KL, Wolf RE Jr.Genetic evidence for pre-recruitment as the mechanism of transcription activation by SoxS of Escherichia coli: the dominance of DNA binding mutations of SoxS. J Mol Biol. 2004 Nov 12;344(1):1-10.

Hidalgo E, Demple B.Spacing of promoter elements regulates the basal expression of the soxS gene and converts SoxR from a transcriptional activator into a repressor. EMBO J. 1997 Mar 3;16(5):1056-65.

Eaves DJ, Ricci V, Piddock LJ.Expression of acrB, acrF, acrD, marA, and soxS in Salmonella enterica serovar Typhimurium: role in multiple antibiotic resistance. Antimicrob Agents Chemother. 2004 Apr;48(4):1145-50.

Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE.The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. Mol Microbiol. 1996 Jan;19(1):101-12.

Hooper DC, Wolfson JS: Mechanism of quinolone action and bacterial killing, in Hooper DC, WolfsonJS (eds): Quinolone Antimicrobial Agents, ed 2. Washington DC, American Society for Microbiology: 53 - 75, 1993.

Boothe, Dawn. *Small Animal Clinical Pharmacology*. 2. St. Louis, Missouri: Elsevier, 2012.154-163. Print.

Kato, J.-I., H. Suzuki, and H. Ikeda**.** 2004. Purification and characterization of DNA topoisomerase IV in *Escherichia coli*. J. Biol. Chem. 267:25676–25684.

Levy SB. From tragedy the antibiotic era is born. In: Levy SB, ed. The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers, 2nd ed. Cambridge, MA: Perseus Publishing; 2002.pp. 1–14

Levy SB, Marshall B. Antibacterial Resistance Worldwide: causes, challenges and responses. Nat Med2004;10:S122–S129

Walsh C. Antibiotics:Actions, Origins, Resistance. Washington, D.C:ASM Press; 2003:3-49

Schwarz S, Chaslus-Dancla E. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Vet Res. 2001 May-Aug; 32(3-4):201-25.

Seguin MA, Vaden SL, Altier C, Stone E, Levine JF. Persistent urinary tract infections and reinfections in 100 dogs (1989-1999). J Vet Intern Med. 2003 Sep-Oct;17(5):622-31.

Cohn, L.A., Gary, A.T., Fales, W.H., Madsen, R.W., 2003. Trends in fluoroquinolone resistance of bacteria isolated from canine urinary tracts. J. Vet. Diagn. Invest. 15, 338–343.

Cooke, C.L., Singer, R.S., Jang, S.S., Hirsh, D.C., 2002. Enrofloxacin resistance in Escherichia coli isolated from dogs with urinary tract infections. J.Am. Vet. Med. Assoc. 15, 190–192.

Johnson JR, Stell AL, Delavari P, Murray AC, Kuskowski M, Gaastra W. Phylogenetic and pathotypic similarities between Escherichia coli isolates from urinary tract infections in dogs and extraintestinal infections in humans. J Infect Dis. 2001 Mar 15;183(6):897-906.

Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother. 2004 Aug;54(2):321-32.

Domagala JM. Structure-activity and structure-side-effect relationships for the quinolone antibacterials. J Antimicrob Chemother 1994;33:685-706.

Zhao X, Xu C, Domagala JM, Drlica K. DNA topoisomerase targets of the fluoroquinolones: a strategy for avoiding bacterial resistance. Proc Natl Acad Sci USA1997;94:13991-6.

Giraud E, Leroy-Sétrin S, Flaujac G, Cloeckaert A, Dho-Moulin M, Chaslus-Dancla E. Characterization of high-level fluoroquinolone resistance in Escherichia coli O78:K80 isolated from turkeys. J Antimicrob Chemother. 2001 Mar;47(3):341-3.

Conrad S, Oethinger M, Kaifel K, Klotz G, Marre R, Kern WV. gyrA mutations in high-level fluoroquinolone-resistant clinical isolates of Escherichia coli. J Antimicrob Chemother. 1996 Sep;38(3):443-55.

Bagel S, Hüllen V, Wiedemann B, Heisig P. Impact of gyrA and parC mutations on quinolone resistance, doubling time, and supercoiling degree of Escherichia coli. Antimicrob Agents Chemother. 1999 Apr;43(4):868-75.

Everett MJ, Jin YF, Ricci V, Piddock LJ. Contributions of individual mechanisms to fluoroquinolone resistance in 36 Escherichia coli strains isolated from humans and animals.Antimicrob Agents Chemother. 1996 Oct;40(10):2380-6.

Ozeki S, Deguchi T, Yasuda M, Nakano M, Kawamura T, Nishino Y, Kawada Y. Development of a rapid assay for detecting gyrA mutations in Escherichia coli and determination of incidence of gyrA mutations in clinical strains isolated from patients with complicated urinary tract infections. J Clin Microbiol. 1997 Sep;35(9):2315-9.

White DG, Piddock LJ, Maurer JJ, Zhao S, Ricci V, Thayer SG. Characterization of fluoroquinolone resistance among veterinary isolates of avian Escherichia coli. Antimicrob Agents Chemother. 2000 Oct;44(10):2897-9.

Huguet A, Pensec J, Soumet C. Resistance in Escherichia coli: variable contribution of efflux pumps with respect to different fluoroquinolones. J Appl Microbiol. 2013 May;114(5):1294-9

Paulsen IT. Multidrug efflux pumps and resistance: regulation and evolution. Curr Opin Microbiol. 2003 Oct;6(5):446-51.

Blair JM, Piddock LJ. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update.Curr Opin Microbiol. 2009 Oct;12(5):512-9

Nunoshiba T, Demple B. Nucleic Acids Res. .A cluster of constitutive mutations affecting the C-terminus of the redox-sensitive SoxR transcriptional activator. 1994 Aug 11;22(15):2958-62

Storz G, Imlay JA. Oxidative stress. Curr Opin Microbiol. 1999 Apr;2(2):188-94.

Amábile-Cuevas CF, Demple B. Molecular characterization of the soxRS genes of Escherichia coli: two genes control a superoxide stress regulon. Nucleic Acids Res. 1991 Aug 25;19(16):4479-84.

Maneewannakul K, Levy SB. Identification for mar mutants among quinolone-resistant clinical isolates of Escherichia coli. Antimicrob Agents Chemother. 1996 Jul;40(7):1695-8.

Ince D, Hooper DC Mechanisms and frequency of resistance to gatifloxacin in comparison to AM-1121 and ciprofloxacin in Staphylococcus aureus. Antimicrob Agents Chemother 2001 45:2755–2764

Kowalski RP, Dhaliwal DK, Karenchak LM, Romanowski EG, Mah FS, Ritterband DC, Gordon YJ Gatifloxacin and moxifloxacin: an in vitro susceptibility comparison to levofloxacin, ciprofloxacin, and ofloxacin using bacterial keratitis isolates. Am J Ophthalmol 2003 136:500–505

Wetzstein HG Comparative mutant prevention concentrations of pradofloxacin and other veterinary fluoroquinolones indicate differing potentials in preventing selection of resistance. Antimicrob Agents Chemother 2005 49:4166–4173