CHARACTERIZATION OF *CAMPYLOBACTER JEJUNI* RESPONSE TO THE MUTANT PREVENTION CONCENTRATION HYPOTHESIS

by

JOAQUIM EUSTAQUIO DE SOUZA AMADO

(Under the Direction of Corrie C. Brown and Paula J. Fedorka-Cray)

ABSTRACT

In this age of antimicrobial resistance concerns, a new hypothesis has been proposed that could serve to increase the lifespan of our antimicrobial stock. It is termed the mutant prevention concentration (MPC) hypothesis, and it states that in every interaction between microbe and antibiotic there is a concentration that will prevent first step spontaneous mutants from appearing after approximately 10^{10} CFUs of bacteria are exposed to the drug of choice. Additional research is needed before this hypothesis could be useful in practical determinations to help establish day-to-day treatment of bacterial infections both in humans and animals. There are many factors, beside the classical and known antimicrobial targets, that are involved in antimicrobial resistance acquisition and facilitation of such events.

Approximately 10^{10} CFU of *Campylobacter jejuni* 81116 and three laboratory derived mutants carrying insertional deletions respectively at *cmeB* (efflux pump), *cmeR* (regulator of the efflux pump) and one presenting a Thr-86-Ile mutation in the *gyrA* gene, were exposed to 2-fold increasing concentrations of nalidixic acid, norfloxacin, enrofloxacin and ciprofloxacin. Mutation frequencies were recorded at each concentration along with the mutant prevention concentration (MPC), and minimum inhibitory concentrations (MICs) for isolated colonies

selected over several trials. Susceptibility results (MICs) were also recorded for each isolate before exposure to each antimicrobial. Susceptibility measurements before exposure of the parent and its transformants to each fluoroquinolone showed a 2-fold decrease for the *cmeB* DNA sequence deleted transformant. Additionally, the absence of *CmeB* protein decreased the MPC consistently by 2-fold for every fluoroquinolone tested. This protein absence also translated into statistically significant mutation frequency decrease. When *CmeB* protein was over-expressed in the *cmeR* sequence insertionally deleted transformant, there were no apparent impacts on MPC or on mutation frequencies.

The data from these studies reveal how MPC could be used as a tool to predict the usefulness of an antimicrobial coupled with the MIC methodology. It also shows that MPC could be another tool for deciding which dose of an antimicrobial to use and which antimicrobial is the one of choice.

INDEX WORDS: mutation frequency, mutant prevention concentration, MPC, fluoroquinolones, *Campylobacter jejuni*, antimicrobial resistance, *in vitro*, mutant selection window, MSW, *gyrA*, *cmeABC*, *cmeR*.

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DEDICATION

This dissertation is dedicated to my parents Hilda Maria Eller and Fernando de Souza Amado. Without their utmost valorization of education, this opportunity of achieving a higher education degree outside Brazil would be impossible. Their admiration for knowledge instilled in me the curiosity and perseverance necessary for a researcher. More than parents they were my mentors through out my young life. I thank God for giving me Fernando and Hilda as parents.

I also would like to dedicate this dissertation to my wife Flavia Teixeira Cezar Amado who sacrificed a few years of her life to follow me in this adventure and who lifted my spirit when I was feeling down.

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CHAPTER 1

INTRODUCTION

The genus *Campylobacter*[1], first described in 1963, is characterized as mostly motile thermotolerant, microaerophilic, slim spirally curved rods. Organisms in this genus are regarded as one of the main causes of bacterial foodborne illness in the USA[2]. *Campylobacter* is found in the intestinal tract of warm blooded animals and is subsequently passed through or released from the intestinal tract to contaminate the environment, including food animals, often during the process of slaughter and processing of meat, especially poultry[3-7].

Campylobacteriosis is generally known as an acute self-limiting diarrhea, rarely progressing to a septic state; as a result, illness is short and antimicrobial treatment is not indicated[8]. However when individuals are young, elderly or immunocompromised, therapy is more likely indicated with macrolides and fluoroquinolones as the typical antimicrobial(s) of choice[9]. Therefore, resistance development in *Campylobacter* to either antimicrobial can quickly become a serious public health problem[10]. Due to this concern, approval for sarafloxacin and enrofloxacin (both fluoroquinolones) use in poultry was withdrawn by the FDA, in order to protect these antimicrobials for use in human medicine[11]. It is likely that other antimicrobials used in animals will most likely face the possibility of having their approval withdrawn on the same grounds. However, although chemistry and generic pharmacokinetics of most antimicrobials is well understood, there is often inadequate scientific data regarding antimicrobial dosage and *in vivo* microbial responses, particularly in animals[12,13]. Many different resistance mechanisms have been characterized and their molecular origins (the responsible genes) have been discerned. However, there are still many indirect and even direct mechanisms to be understood. For instance, in *Campylobacter* there is a mutation frequency decline gene

(*mfd*), which was recently identified as important for development of fluoroquinolone resistance[14]. This gene is capable of promoting mutation frequency via its coded protein *Mfd* under a fluoroquinolone challenge. With an increased mutation frequency *Campylobacter* will more readily develop *gyrA* point mutations that would confer resistance to the fluoroquinolones. The influence of antimicrobial exposure is being actively investigated in a variety of microorganisms by use of microarrays[15-19]. In order to extend the useful-life of antimicrobials for the treatment of humans and animals alike, professional associations such as the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (IDSA)[20], as well as the American Veterinary Medical Association (AVMA)[21], are advocating responsible use of antimicrobials by health professionals in the U.S. Under constant debate is the concern that the use of antimicrobials in food animals will cause the development of resistant strains of bacteria that will impact human health and antimicrobial options will be limited. A class of antimicrobials of particular interest are the fluoroquinolones. These are active against a wide variety of Gram negative aerobes and are frequently prescribed in human medicine. Some fluoroquinolones have been withdrawn from the approved list of antimicrobials for food animals because of resistance development concerns.

Nalidixic acid was the first quinolone developed which exhibited modest activity against some Gram negative bacteria[22,23]. Fluoroquinolones are different from nalidixic acid through the addition of fluorine at the C-6 position[22,23]. Besides the C6-fluorine, norfloxacin also has a piperazinyl ring at C-7 and a nitrogen substitution at the C-8[22,23]. Ciprofloxacin was created by adding an N-1 cyclopropyl group to the norfloxacin basic structure, and this resulted in a much broader spectrum antimicrobial with enhanced activity[22,23]. Enrofloxacin is similar to ciprofloxacin except that for enrofloxacin there is a methylation of the N-2 hydrogen of the

piperazinyl ring[22]. Apparently such methylation can be easily converted in the liver leaving ciprofloxacin as a common metabolic residue of enrofloxacin[22].

As new fluoroquinolones were created by the addition of radicals to the basic structure, the new representatives of the class became more active against Gram-positive bacteria[22]. There is evidence of fluoroquinolone resistance against numerous *Campylobacter* isolates. The mechanisms of resistance include both point mutations of the DNA gyrase gene and expression or over-expression of the efflux pump *CmeABC*. Point mutations on the quinolone resistance determining region (QRDR) of the *gyrA* subunit are capable of altering this subunit conformation, diminishing its affinity to fluoroquinolones[23]. The expression of the *CmeABC* efflux pump synergistically allows the maximum expression of the highly resistant mutation of *gyrA*[24]. In the few over-expressing reports of the *CmeABC* efflux pump[25,26], a point mutation of the *CmeR* (the efflux pump regulator gene) binding site on the intergenic region of *cmeR-cmeA* has been implicated in this increased resistance[26].

Several recent studies involving bacteria other than *Campylobacter* have used a novel methodology known as mutant prevention concentration (MPC) as a means for studying mutations. MPC is defined as the concentration capable of inhibiting the growth of resistant bacterial sub-populations when 10^{10} bacterial cells are exposed to an antimicrobial[27]. Such methodology has been useful for studying many different human pathogens both *in vitro*[28-32] and *in vivo*[33-35]. If an effective MPC can be used therapeutically, then there should be decreased numbers of resistance strains generated, and so it may provide another avenue for combating the overall development of resistance[27].

The aim of this study was to examine the response of *Campylobacter jejuni* strain 81116 and isogenic variants (3 laboratory derived mutants – *cmeB*-, *cmeR*- and a *gyrA* Thr-86-Ile) to each of four different drugs (nalidixic acid, norfloxacin, ciprofloxacin, enrofloxacin), using MPC methodology, and to compare these responses with Minimal Inhibitory Concentration (MIC) data, the most widely used clinical measurement used for determining resistance.

The dissertation is separated into four chapters. Chapter 2 is a review of the available, searchable knowledge about *Campylobacter* infections in humans, the treatment, and the mechanisms of fluoroquinolone resistance. Chapter 3 describes an *in vitro* study where 4 variants of the same strain were exposed to different concentrations of 4 different fluoroquinolones and determination of mutation frequencies, of MPCs, and recovered MICs was the objective of our analyses. Chapter 4 is a comprehensive discussion of the conclusions and recommendations for future research efforts in the subject.

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CHAPTER 2

LITERATURE REVIEW

History and taxonomy

The genus *Campylobacter* was originally classified in the *Vibrio* genus and was first identified as a pathogen related to abortion in sheep and cattle (caused by *Campylobacter fetus* subsp. *fetus*)[1]. Currently the genus includes approximately 16 different species, with two, *Campylobacter lanienae* sp. nov. and *Campylobacter hominis* sp. nov., having unresolved taxonomic status [1-5]. *Campylobacter jejuni* and *Campylobacter coli* are recognized as the two species of public health concern in the foodborne gastroenteritis complex of diseases, and when first isolated, were called *Vibrio jejuni* and *Vibrio coli,* respectively[1].

The name *Campylobacter* was proposed in 1963 to describe a group of isolates of the *Vibrio* genus that presented a lower DNA G:C ratio when compared to the other members. In addition to this G:C difference, a microaerophilic atmosphere requirement that differed from the facultative anaerobic characteristics of the *Vibrio* genus, and lack of the genus-recognized fermentative ability also supported the change[6]. In the latest taxonomy review Vandamme *et al* 1991[5] performed DNA-rRNA hybridizations of all *Campylobacters* and related taxa and determined that they are part of the phylogenetic group named rRNA superfamily VI. Inside this superfamily the authors proposed three rRNA homology groups called clusters I through III, with all species of *Campylobacter* included on cluster I along with *Bacteroides ureolyticus*[5]. Because of their phenotypic differences in proteolytic metabolism and fatty acids components, researchers believe that further studies of diversity are necessary to move *B. ureolyticus* to the genus *Campylobacter*[5,7].

Campylobacter are slim, spirally curved rods with a width between 0.2 and 0.8µm and a length between 0.5 and 5µm[1]. For cultures older than 48h, or when bacteria are stressed, the morphology may change to the coccoid form[8,9]. *Campylobacter* are chemoheterotrophic, non-sporeforming, gram-negative bacteria that are microaerophilic and do not tolerate strict aerobic or anaerobic environments[1,10]. Most of the species and strains are motile and have a distinctive corkscrew-like motion when viewed microscopically which is attributed to its curved morphology; a single unsheathed flagellum polarized is found at one or both ends[1,10]. Colonies on blood-agar plates are gray, non-hemolytic, and mucoid[11]. *Campylobacter* spp. may be reliably identified by their shape, Gram stain and positive result in the oxidase test. Other common characteristics within the genus are catalase presence, nitrate reduction and growth at 42°C[10]. The *C. jejuni* subspecies can be differentiated biochemically, with *C. jejuni* subsp. *doylei* characterized as incapable of nitrate and selenite reduction; they are also unable to grow at 42°C while *C. jejuni* subsp. *jejuni* are capable of such growth[1,10,12,13].

Human campylobacteriosis: clinical symptoms and treatments.

Only over the last three decades has *Campylobacter* has been acknowledged as an important enteric pathogen in humans. *Campylobacter jejuni* subsp. *jejuni*, *C. coli*, *C. lari*, *C. uppsaliensis and C. jejuni* subsp. *doylei* are recognized as enteropathogenic to humans[14]. *Campylobacter jejuni* subsp. *jejuni,* usually referred to simply as *Campylobacter jejuni*, is responsible for most of the reported human cases of campylobacteriosis[15].

Campylobacter is considered one of the main causes of bacterial foodborne illnesses in the USA[16] and the Centers for Disease Control and Prevention (CDC) has estimated that 2.4 million people or 0.8% of the general population can be clinically ill with *Campylobacter*

infections every year[17]. The latest report from the Foodborne Diseases Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program has found no change in the incidence of campylobacteriosis when comparing 2007 data with the period from 2004 to 2006[16]. According to the FoodNet active surveillance, a total of 5,818 culture positive cases were reported, substantiating 12.79 cases per 100,000 Americans[16]. *Campylobacter jejuni* is found in the intestinal tract of many species of warm blooded animals, insects, and in water collections that receive runoff from animal holding facilities[12,18-21]. In developed countries, the most important source of infection for humans is recognized to be poultry meat, although many other sources of infection exist[22-29]. Cases of human campylobacteriosis in the community most often appear as sporadic and rarely in the form of outbreaks, particularly in the industrialized nations[30,31].

The prevalence of *Campylobacter* on poultry carcasses and other raw retail cuts varies widely (table 2.1). This is due in part to producer variability, seasonal variability, and lack of a consistent methodology of sampling and culture. Therefore, it is difficult to record an accurate estimate of *Campylobacter* prevalence in chicken meat within a country and especially worldwide. Table 2.1 lists some data collected from the literature regarding prevalence in differing countries. In a six-year survey in Ireland samples of raw retail chicken were separated by producers. There were substantial differences in contamination among different producers, demonstrating that the origin of the chicken sample is important in such a survey[32]. In a year-long survey in the U.S., 30 samples were taken monthly from retail market poultry, and the recovery during the period from May to October was highest[33]. An article from Bulgaria demonstrates the possible influence of storage temperature (frozen or chilled) and poultry meat contamination rate with *Campylobacter spp* [34]. Infection of humans is most likely due to

consumption of undercooked poultry[27] or cross-contaminated food and kitchen utensils[35]. A case-control study from FoodNet sites determined that eating poultry products prepared in a commercial establishment presented the higher risk of infection[27]. In yet another study that used Monte Carlo simulations to determine the cross-contamination probability of salads via kitchen surfaces, the report showed that it is realistic to expect such risk factor as part of a epidemiological pathway[35].The infectious dose for humans may be as low as 500 organisms[36,37].

Clinical signs of *Campylobacter* infection in humans include diarrhea, abdominal pain, headache and fever[36-38]. Such symptoms are not specific to campylobacteriosis alone and can occur with a number of gastrointestinal infections. Therefore without culture and isolation of the etiologic agent from the patient's stool it is almost impossible to point out the differences from many other etiologic causes.. *Campylobacter* enteritis is usually a self-limiting disease, which rarely undergoes systemic spread. This limitation has been attributed to its sensitivity to the blood complement- and antibody-mediated immune defenses[39].

Because of their broad-spectrum nature, the antimicrobial agents of choice for treatment of complicated campylobacteriosis are fluoroquinolones (mainly ciprofloxacin), and macrolides (mainly erythromycin)[38]. Other antimicrobials such as tetracyclines and aminoglycosides may be used as alternative antimicrobial agents and in the event of complications[38]. Studies have shown a link between antimicrobial resistance in *Campylobacter* species and adverse health events in humans including a longer infection time and resistance to fluoroquinolone treatment[40-43].

Complications may occur in immunocompromised patients, children and elderly people and may result in one or more of the following: bacteremia[44], hemolytic uremic syndrome[45,46], toxic mega-colon[43], reactive arthritis[47], or Guillain Barré syndrome (acute neuromuscular

paralysis)[48,49]. FoodNet data for 2007 shows a high incidence of campylobacteriosis in children aged <5 years (24.01 per 100,000 population)[16].

Commensal colonization of birds

Campylobacter spp. are cultured from many avian species such as emu, hawk, ostrich, and parrot[50]. Broiler chickens can become infected early in life, and by using microbiological techniques, colonization is detected from 2 to 4 weeks of age[51]. By PCR (16S ribosomal DNA PCR), detection of colonization has been recognized as early as 4 days of age [52].

Studies of the microbial succession in broiler intestines examined by analysis of several 16S rDNA partial genes demonstrates that the intestinal microbial flora exhibits two stages of stability - first, at 2 to 4 weeks (14 to 28 days) and then again at the end of the grow-out (49 days)[53]. The same study concluded that at 3 days-of-age the unique microbiota is soon substituted by a more mature and stable bacterial community, presenting a unique opportunity for colonization by bacterial pathogens such as *Campylobacter*[53,54]. This hypothesis was better tested in a similar study with preadolescent turkeys that showed indigenous microbiota instability at 11 and 12 weeks coincided with a bloom of *Campylobacter coli* [54]. Microflora succession studies generally start by providing a steady feed formulation which may not change. This is not the case in commercial production of poultry where feed ingredients change at different stages in the broilers production and at other times due to different quality or budget constraints. A change in quality of the feed ingredients, or in the ingredients themselves, is capable of affecting the chicken intestinal indigenous microbiota and offers a temporal window for colonization by pathogens of zoonotic interest like *Salmonella enteritidis*, *Campylobacter spp*., *Clostridium*

perfringens, *E. coli*, and *Staphylococcus aureus*[53,55,56]. In a study of 8 American farms with a total of 32 flocks for a year, 87.5% of the flocks became infected by *Campylobacter*[57].

Once introduced into a few birds, the spread within the flock is rapid[58]. The carriage of *Campylobacter* through production to the final poultry meat cuts has been confirmed with the help of pulse field gel electrophoresis(PFGE)[59]. When positive birds are slaughtered all surfaces of the bird may be positive. Therefore, slaughter plant environmental contamination, with subsesequent cross-contamination of negative carcasses, is common during processing operations[51]. Some of the logical critical control points where negative broilers carcasses and external surface of positive broilers may become contaminated are the scald tank, feather picker in the evisceration line (specifically the vent-cutter) and chill water[57,60,61]. According to a recent research article the picker could be one of the most important points for externalization of *Campylobacter* from the intestinal contents[62].

After poultry meat is contaminated with *Campylobacter*, the bacteria can remain on the meat as long as the poultry shelf-life. In a study, strain 81116 (same one that was used in this study) was inoculated onto chicken skin and submitted to various temperatures (25, 4, -20, -70°C) and different packing procedures[63]. At all freezing levels *Campylobacter* was able to survive and grow to infective levels after thawing[63]. Replication occurred at both at 4 and 25°C[63]. In a different study, thermal death times (D-values) were determined in sterilized ground chicken meat and 1% peptone broth inoculated with a *Campylobacter* suspension, showing that it can be easily deemed unrecoverable, and probably incapable of causing human infections after normal cooking procedures[64].

Although normally not pathogenic for birds, *C. jejuni* has been linked to the condition called vibrionic hepatitis in which liver lesions of a yellow to white radiant appearance present in

a few foci of the chicken liver; this may be seen during inspection after slaugher[65,66]. Such lesions were also recorded in ratite chicks in normal field conditions and in Japanese quail under laboratory conditions[67,68]. The condition was mostly recorded in laying hens during the 50's and 60's, affecting 10% of the birds in a commercial laying house. This contributed to lowering egg production up to 35%, and an increase in the normal production mortality of 9 to 10%[66]. No effective treatment against the infection has been reported, but protocols that use chlortetracycline or tiamulin have been proposed[66]. Diarrhea is generally not associated with *Campylobacter* infection in poultry, but there are rare reports in the literature that associate it with diarrhea[69,70].

Antimicrobial resistance

Antimicrobials are used in animal production both therapeutically and non-therapeutically[71-73]. Dosages, route or vehicle of administration vary according to the drug and its intended use[71-73]. Drugs used with therapeutic purposes are given to individual animals or groups in a dose high enough to promote clinical cure and elimination, or at least inhibition, of the etiological agent[71]. Metaphylaxis is characterized by the administration of short duration therapeutic treatment to a group of mixed healthy (larger number) and sick (smaller number) animals, aiming towards prophylactic treatment of the healthy animals and therapeutic treatment of the sick[71]. Prophylactic administration may be done at therapeutic or subtherapeutic dosages, is often given in feed or water for a longer time frame when compared to metaphylaxis and is typically reserved for clinically normal animals that are likely to encounter conditions favorable to acquisition of infectious agents[71]. Antimicrobials used to increase feed efficiency, most often

referred to as growth promoters, are given at subtherapeutic concentrations as feed or water additives in order to promote weight increase in a smaller time frame[71-73].

Both therapeutic and subtherapeutic antimicrobial dosages are able to elicit selection of resistant bacteria[74-77]. Resistance to antimicrobials occurs in various ways: as selection for, and enrichment of, pre-existing resistant isolates; as selection of isolates that have undergone random mutation which resulted in resistance; through transformation or transference of genetic sequences such as plasmids, phages, mosaic genes, and transposons; and/or by induction of pre-existing but somehow dormant biochemical pathways and enzymes[72].

Campylobacter behaves as a commensal bacterium of the chicken gastrointestinal tract and is only rarely pathogenic in poultry[78-80]. Therefore no antimicrobials have been developed which are indicated for treatment of *Campylobacter* in poultry. Fluoroquinolones (sarafloxacin and enrofloxacin) were used to treat *Escherichia coli* infections, decreasing related mortality and carcass-damaging lesions such as air sacculitis, pericarditis, perihepatitis[74]. During production or clinical treatment, antimicrobials are administrated simultaneously to all the animals within a chicken house mainly through feed or water[71,73]. The voluntary withdrawal of sarafloxacin published on April 30th of 2001 in the Federal Register[81] left enrofloxacin as the only licensed fluoroquinolone for use in poultry production. However on July 27th, 2005, the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) withdrew approval for its use and determined that the use of fluoroquinolones causes development of fluoroquinolone resistant *Campylobacter*, posing a risk of transference to humans and therefore a hazard to public health[82]. Data was presented which showed that *Campylobacter* easily acquired resistance to fluoroquinolones without any viability cost (without any deleterious change to their growth rate or survival mechanisms) when chickens were treated against colibacillosis (the target organism)

with sarafloxacin and particularly with enrofloxacin [74,75,83-85]. In a study in Minnesota 6 out of 7 PCR-RFLP characterized strains with antimicrobial resistance were isolated from poultry carcasses and human stool samples alike showing a very probable epidemiological interaction that could be supported by this molecular data[86].

Fluoroquinolone resistance has also developed when humans infected with *Campylobacter* are medicated with fluoroquinolones[87,88]. Such correlation is generally regarded as being of lesser importance because human to human transmission is somewhat rare[89] although not impossible[90,91]. Temporal correlations between the increase in fluoroquinolone resistance and approval of drugs from this same family to be used in animal production has been discussed[92].

In the U.S. antimicrobials are subject to approval by the FDA before license and marketing. As part of this approval, breakpoints are also established by the FDA based on data submitted by the production company[93]. The Clinical and Laboratory Standards Institute (formerly NCCLS) reevaluates those breakpoints along the commercial life of the drug, taking into account the following: population distribution of Minimal Inhibitory Concentrations (MICs) related to known resistance mechanisms; the relationship between MICs and the clinical outcome coupled with the bacteriology; and the pharmacodynamics of individual drugs[94]. However, during the establishment of MIC breakpoint guidelines, there is no accounting (at anytime during or after approval) for the frequency or rate of mutations that may occur when bacteria are exposed to a particular drug[93,94].

To minimize or prevent the development of resistance, it may be necessary to change the current strategy from simply targeting clinical cure to actually blocking the recovery of mutants. It will at least be important to understand the influence of the interaction of microorganisms, antimicrobials and infected host to resistance acquisition by the infecting pathogen.

Fluoroquinolones activity

Around five decades ago nalidixic acid, a completely synthetic antimicrobial, was introduced and although it presented limitations that made it suitable only for the treatment of uncomplicated urinary tract infections of humans (UTIs) caused especially by *Escherichia coli,* it paved the way for the development of the fluoroquinolones[95].

The chemical structure of nalidixic acid is a naphthyridone bearing a nitrogen atom in place of carbon at position 8[96]. Its primitive quinolone chemical structure was later modified to allow a broader spectrum of activity, better clinical pharmacokinetic characteristics, lower toxicity and decreased development of resistance[97]. Nalidixic acid was and is still used by many laboratories to differentiate between *Campylobacter lari,* an intrinsically resistant species, and *Campylobacter jejuni* and *C. coli* which are originally sensitive. Several fluoroquinolones were then developed from the nalidixic acid basic structure but only a few are of veterinary interest, and less than that are concomitantly of human medicine importance.

Unlike nalidixic acid, norfloxacin was readily absorbed presenting a higher blood serum half-life, lower protein binding characteristic, and the capability to rapidly penetrate inflammatory sites[95,98]. The structure of norfloxacin contains as a central figure the naphthyridone with a fluorine at position C-6 in place of a hydrogen atom, and a piperazinyl radical at C-7[95,96]. Norfloxacin was the first broad spectrum fluoroquinolone marketed in 1986 for use in the U.S. Ciprofloxacin is similar in structure to norfloxacin; their difference is noted at the N-1 of the naphthyridone basic structure where instead of an ethyl radical there is a cyclopropyl[97]. Ciprofloxacin usage in human medicine and indications varies with its form of administration (oral or intravenous). It is used to treat urinary tract infections, lower respiratory tract infections, skin and skin structure infections, bone and joint infections, infectious diarrhea

and typhoid fever (FDA; NDA#019537, 019847, and 020780 and related labels)[99]. These usage indications are much broader than those permitted for the previous quinolones and fluoroquinolones. Enrofloxacin, a fluoroquinolone exclusively used in veterinary medicine especially poultry production, has basically the same structure of ciprofloxacin except for an ethyl addition on the piperazinyl ring[96]. Because enrofloxacin is readily metabolized in the liver of animals to ciprofloxacin, indirect exposure of animals to ciprofloxacin occurs when they are medicated with enrofloxacin[100-103], although ciprofloxacin is not approved for animal use in the U.S. (table 2.2). Many other fluoroquinolones have been produced after norfloxacin, but just a few have been suggested for both veterinary and human clinical use (table 2.2).

Quinolone and fluoroquinolone basic mechanisms of action are the inhibition of the DNA replication by jamming it. As the replication fork goes forward the DNA portion ahead is positively supercoiled while a negatively supercoiled DNA is left behind[104]. Topoisomerase II, also called gyrase, and topoisomerase IV are responsible for unwinding and winding the DNA double helix so that the replication fork can move between the several nucleotides that are the replication target[104]. The gyrase is divided in two subunit proteins *GyrA* and *GyrB*. Toposisomerase IV is divided into *ParC* and *ParE* subunit proteins. Many articles have reported that *Campylobacter* presents only the *gyrA* DNA seqence[105-109] although one report has identified *parC* by PCR amplification and subsequent sequencing[110]. At present, the *parC* gene has not been identified in any *Campylobacter* species sequenced and loaded on the National Center for Biotechnology Information (NCBI) website[111].

Fluoroquinolone resistance

Fluoroquinolone resistance mechanisms in *Campylobacter* include either mutations of the DNA gyrase gene or activation of an efflux pump. A very small amount of isolates may have neither of these mechanisms and may still be resistant to ciprofloxacin at levels such as 16 and 32mg/L or even resistant to multiple antibiotics[108,112].

Point mutations in the quinolone resistance determining region (QRDR) at the *gyrA* subunit, translates into amino acid substitutions at Thr-86-Ile[113-118], Thr-86-Ala[109,119,120], Thr-86-Lys[106], Asp-90-Asn[106,109,119,121], Ala-70-Thr[121], Pro-104-Ser[108,118,119], Ser-22-Gly, Asn-203-Ser, and Ala-206-Thr[122]. The mutation at position 86, particularly the Ile substitution, is the most common mutation observed in field and *in vitro* isolates, from humans and animals alike[105,107,108,117,121,123]. Substitutions in these regions correlate with the ability to resist the effects of the fluoroquinolones. Each one of the above point mutations will, in different degrees, lower the affinity of the type II topoisomerase to the fluoroquinolones, avoiding the formation of DNA/gyrase/antimicrobial complexes that “jam” the DNA replication fork[97]. However no one has been able to predict how much the susceptibility to fluoroquinolones will be lowered by each mutation. Table 2.3 lists a collection of articles that present MICs and sequenced QRDR and highlights the variability of this relationship. Of course such a generic table does not account for methodology used to measure the MICs, but only records the lowest and highest limits of the data while giving us an idea of the variability. A good and reproducible example of such variability can be seen in the article of Piddock *et al* 2003[108] where the authors looked at 213 isolates. From these 213 isolates, 171 presented the Thr-86-Ile mutation while their ciprofloxacin MICs varied from 2 to 128µg/ml[108]. From the same article it is shown that even the susceptible isolates are equally variable, presenting with ciprofloxacin sensitivity results that vary from 0.12

to 32µg/ml[108]. Low susceptibility to a particular fluoroquinolone due to *gyrA* point mutations may or may not result in cross-resistance to other fluoroquinolones depending on the affected codon position and resulting mutated amino acid[120]. However, clinically relevant cross-resistance to unrelated antibiotics is not observed.

Unlike other Gram-negative bacteria, *Campylobacter* presents the gyrase gene as the only target for the fluoroquinolone's action; therefore the so called first step mutation happens in the *gyrA* gene as well as all the subsequent steps[107,124]. Another distinguishing feature in *Campylobacter* is that the mutation carrying the higher resistance outcome, Thr-86-Ile[107], is easily selected in the first-step of the mutation and subsequent steps may increase resistance by adding different significant amino acid changes such as Asp-90-Asn[107,125]. One article has examined this multiple mutation paradigm of *Campylobacter* at positions 86 and 90 of the *gyrA* QRDR[125]. By following the molecular Koch's postulate they were able to identify the influence of deletion and recovery of a codon change at the 86 position of *gyrA*[125]. When a Thr-86-Ile mutation was introduced in the sensitive parent strain genome, an increase of 128-fold and 64 to 32-fold were recorded for ciprofloxacin and nalidixic acid, respectively[125], while the other point mutation influence at position 90 was asserted by the difference between its presence (as a secondary mutation) and its deletion by insertion of only the 86 position change in place of the double mutations[125]. However additional data is needed before it can be determined whether this secondary change happened before, after or at the same time of the main Thr-86-Ile mutation. All the other point mutations are still pending such molecular Koch's postulate style proof.

Efflux pumps in *Campylobacter jejuni* were linked not only to fluoroquinolone resistance, but to multidrug resistance as well[125,126]. A tripartite multidrug efflux pump (TMEP) was characterized in *C. jejuni* 81-176 and NCTC11168 by Lin et al in 2002[127] and Pumbwe and

Piddock (2002), respectively[128]. The TMEP system is composed of a fusion protein, an efflux pump and an outer membrane channel protein or the *CmeA*, *CmeB* and *CmeC* genes, respectively. *CmeB* is capable of conferring increased resistance to multiple antibiotics and other substances like bile salts, detergents, and dyes. As a result of its role in bile salt resistance, the *CmeABC* is also very important for bacterial colonization of the chicken gut[129]. When this pump was inactivated, isolates could not grow in the presence of 2mM cholate in culture media and the infectious dose in chickens was increased by at least 2.6 X 10^4; however, both of these effects were lost when the pump was reactivated[129]. The efflux pump was also expressed by *Campylobacter in vivo*, in chickens, as demonstrated by immunobloting trials[129]. Multiple antimicrobial resistant (MAR) *Campylobacter* strains were also shown to occur by over-expression of *cmeB* efflux pump, which was recorded in 9 out of 32 MAR isolates examined and these events were linked to a mutation substituting glycine 86 by alanine in the *CmeR* encoding sequence[130].

Recently a MAR isolate resulting from a point mutation in the *CmeR* binding site, was selected by 3 consecutive passages of a Thr-86-Ile *gyrA* mutant in media containing 16µg/ml of enrofloxacin[131]. The development of a *cmeR*-*cmeA* intergenic mutant resulted in 8-fold increases for clindamycin, 4-fold increases for ciprofloxacin, enrofloxacin, tylosin, chloramphenicol, ampicillin, and for tetracycline and 2-fold increases for nalidixic acid, and erythromycin[131]. This increased the awareness of the role played by efflux pumps in antibiotic resistance[131]. It is clear that the pump contributes in its physiological expression level to fluoroquinolone resistance in synergy with *gyrA* point mutations by lowering the MIC to ciprofloxacin 21 to 168-fold when compared to the ciprofloxacin resistant parent strain when *cmeB* was inactivated[106]. Ten different putative efflux pumps were also recognized by an NCBI search within the *C. jejuni* NCTC11168

genome. When parent cells underwent insertional mutagenesis of the *cmeB* efflux pump sequence, they presented a 4 to 256-fold reduction in resistance to ciprofloxacin, erythromycin, tetracycline and chloramphenicol[125]. Greatest increases in susceptibility were noted when previously highly MAR resistant parents underwent *cmeB* insertional deletions[125]. However, experiments performed with efflux pump inhibitor L-phenylalanine-L-arginine-β-naphthylamide (PAβN) have demonstrated a different finding, where MICs were not altered for the highly quinolone resistant *Campylobacter*[107,116,132]. This may indicate that the action of *CmeABC* TMEP on fluoroquinolone resistance is negligible. Interestingly, one group that originally concluded that *CmeABC* was not relevant after using PAβN later reversed their opinions, concluding that no efflux pump inhibitor should be used without proper validation of its effectiveness[132].

A member of the TetR family of transcriptional repressors, *cmeR,* encodes a repressor of the *cmeABC* DNA promoter. Therefore, impairment of either the *cmeR* sequence or inverted repeat will lead to over-expression of the efflux pump conferring multidrug resistance[132,133]. Bile salts are able to enhance the *cmeABC* DNA sequence expression 6 to 16-fold by interfering with the binding of *CmeR* protein to the *cmeABC* promoter[134]. Taurocholate, especially, resulted in a 2 to 4-fold increase in the MICs of cefotaxime, novobiocin, ciprofloxacin, fusidic acid, and erythromycin[134]. Corcoran *et al* 2005[132] found a strain isolated from poultry that presented 12 and 32 mg/ml susceptibility results to ciprofloxacin and nalidixic acid, respectively without any *gyrA* mutation. The authors have demonstrated an enhanced efflux capability of the strain and a point mutation in the *cmeR*-*cmeA* intergenic region that may also be responsible for the low level fluoroquinolone resistant profile[132].

First characterized by Pumbwe *et al* [112] a second efflux pump, which is a member of the resistance nodulation cell division (RND) family, is also related to the MAR-like phenotype in *C.*

jejuni, but it did not transport ciprofloxacin out of the cell. In 2006, Akiba *et al* independently proceeded to conduct insertional mutagenesis of *cmeF* and *cmeB* in different genetically characterized strains (NCTC 11168, 81-176, and 21190) [135]. They concluded that this newly characterized pump would interact with *CmeABC* to confer cell viability and antimicrobial resistance[135].

In view of the fact that it does not matter if the fluoroquinolone resistance originated by an alteration of the *gyrA* or the *cmeABC* DNA sequence (particularly the *cmeR-cmeA* intergenic region), the phenomenon of spontaneous genetic mutation is the key element for both mechanisms. Therefore, determination of mutation frequency after exposure to a fluoroquinolone concentration is important for our understanding of fluoroquinolone resistance. Taylor *et al* showed that concentrations of 64 and 128µg/ml of nalidixic acid did not select for resistant isolates from exposed sensitive *C. jejuni* and *C. coli* strains. However, at 32µg/ml of nalidixic acid in Mueller Hinton agar (MHa), mutants of *C. jejuni*, and *C. coli* arose at frequencies of 2.5 X 10^{-8} and 7.8 X 10^{-9}, respectively, per colony forming unit exposed[136]. Taylor *et al* found that a 4-fold increase in the concentration of nalidixic acid will result in a 10-fold decrease of the chance to select for mutants when one cell is exposed to this quinolone[136]. In a more recent article, *C. jejuni and C. coli* strains had spontaneous mutation frequencies ranging from 4 X 10^{-9} to 7 X 10^{-3}, hence the name hypomutable and hypermutable, respectively[137]. In yet another article the strain NCTC 11168 was transformed into two variants that were either not expressing or over-expressing the *cmeABC* multidrug efflux pump sequence[138]. There was a significant difference in the mutation frequencies presented by the 11168 transformant unable to express the efflux pump (2.54 X 10^{-9}) versus the one over-expressing efflux pumps (3.92 X 10^{-6}) and the wild-type (0.91 X 10^{-6}) when they were exposed to 1.25µg/ml of ciprofloxacin[138]. To the extent

of our knowledge, the data reported regarding *Campylobacter* mutation frequencies were produced by an initial inoculate that had intrinsically little or no standardization concerning the number of cells exposed to the drug. It is plausible then to conclude that those cells could have been exposed to variable amount of nutrients which makes comparisons between experiments, or even within the same experiment, difficult to impossible to compare, since this likely played a role in the mutation frequency differences. Other missing information includes the amount of culture used for plating and the plate size itself, which could have contributed to a less than desirable growth condition due to crowding. The fact that these reports never provided this information leaves us to make assumptions that may not be accurate in the calculation of the mutation frequency.

When strain NCTC 11168 was exposed to ciprofloxacin, microarray analysis indicated that 45 genes exhibited a change in their expression and the gene specific for mutation frequency decline was one of those genes[139]. This gene, named *mfd* for mutation frequency decline, is involved in DNA repair and encodes a transcription-repair coupling factor. The role of *mfd* in *Escherichia coli* is to help the repair of DNA damage by uncoupling the RNA polymerase damaged DNA complex allowing the work of *Uvr*ABC excision-nuclease[140,141]. It is also known to be capable of bypassing translocation of *E. coli* RNA polymerase on damaged DNA[141]. In the study with *C. jejuni* NCTC 11168, this genetic sequence was a big factor in the emergence of spontaneously resistant fluoroquinolone mutants instead of decreasing them[139]. Transformants carrying a mutation that impaired the *mfd* function diminished the frequency of mutation 100-fold. The project was run both *in vitro* and *in vivo* using chickens inoculated with the transformants. In both experiments the result were the same - the impairment of the *mfd* gene

decreased the presence of fluoroquinolone resistant *Campylobacter* after ciprofloxacin exposure[139].

The mutant prevention concentration (MPC)

In 1999 and 2000 the research group of Karl Drlica and Xilin Zhao, after observing the decrease in the recovery of resistant *Mycobacterium bovis* and *Staphylococcus aureus* [142] from plates with increasing concentration of fluoroquinolones defined the limits of what Baquero in 1990 described as a dangerous concentration range where resistant strains are promptly selected[143]. This range was named Mutant Selection Window (MSW) and is defined as the concentration delineated by the minimal concentration able to kill approximately 99% of the cells in the media (MIC_{99}) while the minimal concentration capable of blocking the growth of the least drug susceptible mutant subpopulation (or the single-step mutant) is called the Mutant Prevention Concentration (MPC)[144]. For microorganisms to be able to grow at concentrations equal to or above the MPC they may need to develop multiple mutations or even possibly acquire mobile genetic elements conferring resistance. Interesting enough, using the MPC measurement allows assessment of the concentration needed to avoid antimicrobial resistance development without knowing exactly all the different molecular mechanisms involved[142].

In order to find the MPC, a number of bacterial cells or colony forming units ($\geq 10^{10}$CFU) are exposed to culture media containing increasing concentrations of the antimicrobial of interest[145]. In contrast, when measuring MICs the numbers of CFUs are limited from 10^4 to 10^5 CFU/ml. Such limitation is imposed to diminish any confounding result that could occur due to the presence of resistant subpopulations[146-148]. In MPC such subpopulations are actually the

focus point of the methodology, and therefore their presence is necessary to assess the concentration capable to inhibit their enrichment[145].

Until now the MPC theory has been tested with various microorganisms including: *Staphylococcus aureus* [142,149-158], *Pseudomonas aeruginosa*[159-164] , *Escherichia coli* [157,165-167], *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*[160], *Mycobacterium tuberculosis*[168-170], *Streptococcus pneumoniae*[162,163,171-177], *Haemophilus influenzae*[162,171], *Moraxella catarrhalis*[162,171], *Mycobacterium avium*[178], *Mycobacterium bovis*[142], *M. smegmatis*[158,179], and *Salmonella enterica*[180]. Most tests were conducted *in vitro* using media with added antibiotic or by using pharmacodynamic models. The drugs used most often were the fluoroquinolones, especially because their limited number of known resistance mechanisms is often due to point mutations. Only a few were tested *in vivo*[172,173,181], but it was enough to show a solid correlation vis-à-vis with the *in vitro* results. Although a moderate amount of work has been done, additional testing must be performed and evaluated before it is considered a valid clinical strategy.

If antimicrobial dosages are set up in a way that the drug may reach the target bacteria within its site, in a concentration above the recorded MPC, it is expected that the probability of resistance development will be very low (below 10^{-10} CFU at least)[145]. A limitation of this line of reasoning is that there are many mechanisms for developing antimicrobial resistance, in addition to mutation[145]. The appropriate index for such effect is still under discussion but the most accepted one is the ratio of the area under the concentration time curve by the MPC (AUC/MPC)[145]. This index is supported by two *in vitro* pharmacodynamic/pharmacokinetic (PK/PD) experiments that were run with *Staphylococcus aureus* and *Escherichia coli* against fluoroquinolones[152,182]. In both experiments there was a positive correlation between AUC/MPC

and prevention of resistance development. Two other PK/PD *in vitro* experimental models that were run with *Streptococcus pneumoniae*[172] and *Staphylococcus aureus*[183], also against fluoroquinolones ,correlate the prevention of resistance development with a decreasing amount of time spent at the MSW (T_{MSW}).

Recently a letter to the editor was published that presented the MPC of ciprofloxacin, nalidixic acid and enrofloxacin sensitive isolates of *Campylobacter*[184]. This publication was the first of its kind to analyze the MPC for sensitive and fluoroquinolone resistant *Campylobacter* isolates. To the best of our knowledge no research has been done to determine the influence of the characterized *CmeABC* efflux pump on the MPC activity. Therefore, this body of work serves to provide additional *in vitro* information on the effect of efflux pumps on MPC, while at the same time checking the response of a common fluoroquinolone resistant mutation to this hypothesis.

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Table 2.1 Percent prevalence of retail chicken products contaminated with *Campylobacter* as reported in the literature

Country	**Geographic area**	***C. jejuni*** (%)	***C. coli*** (%)	***C. jejuni/coli*** (%)	***Campylobacter spp*** (%)	***n***[a]
US	[b]ND[33]	69.4				330
	King County, Washington[185]			23.1		862
	Minnesota[86]			14		91
Spain	9 Provinces of Castilla and Leon[186]				49.5	197
UK	[b]ND [187]	68				300
	[b]ND [188]				83	241
Germany	[b]ND [189]				45.9	509
Ireland	Northern Ireland, Belfast[32]				56.9	803
Switzerland	[b]ND [190]				24.38	800
	[b]ND [191]				21.93	415
Bulgaria	[b]ND[34]	57			19.25	135
Japan	Akita prefecture[192]	71.2				73
	Saitama[193]	45.8				72
Vietnam	Hanoi[194]	14	8		9	100
Korea	[b]ND [195]			61.8		923
Australia	New South Wales[196]				87.8	549
	South Australia[196]				93.2	310

Our objective was to report only results for chilled retail chicken products. Articles that surveyed a broader range of foods were included, but only their figures pertaining to chicken retail products were included on the table. Sampling period was not considered while putting this table together, therefore seasonality variation may greatly confound the comparisons of the different reference results.

Some incidences presented above were calculated from the reference provided in order to standardize the unit reported on the table. Reference numbers are provided as superscript on the geographic area column.

[a]Number of samples cultured to find the reported incidence.

[b]Geographic area results with ND (not determined) correspond to an absence of specific geographic citation in the original article.

Table 2.2 Fluoroquinolones approved for human and veterinary clinical use in US by the Food and Drug Administration[197,198]

		Pets		Food Animals			
	Humans	**Dogs**	**Cats**	**Dairy**	**Beef**	**Swine**	**Poultry**
Ciprofloxacin	+	-	-	-	-	-	-
Ofloxacin	+	-	-	-	-	-	-
Norfloxacin	+	-	-	-	-	-	-
Difloxacin	-	+	-	-	-	-	-
Enrofloxacin[a]	-	+	+	+	+	+	-
Danofloxacin	-	-	-	-	+	-	-
Marbofloxacin	-	+	+	-	-	-	-
Orbifloxacin	-	+	+	-	-	-	-
Levofloxacin	+	-	-	-	-	-	-
Gatifloxacin	+	-	-	-	-	-	-
Moxifloxacin	+	-	-	-	-	-	-

The table above only cites the fluoroquinolones that are not only approved, but also being marketed in US.

[a]Enrofloxacin and danofloxacin are the only fluoroquinolones still approved to be used in cattle (dairy and beef) and swine, is not approved to be used as growth promoter.

Table 2.3 Compilation of literature describing comprised sequenced *gyrA* genes and their respective fluoroquinolone sensitivity results

	Observed MIC (μg/ml or mg/L) **ranges**										
Point Mutation	**NAL**	**NOR**	**CIP**	**ENR**	**ORF**	**LEV**	**MOX**	**GRE**	**GAT**	**TRO**	**n**
Thr-86-Ile[83,105-109,113,114,116-123,132,138,199-203]	2 to 256	50 to 128	2 to 128	4 to 128	8 to 32	32	2 to 8	32 to 128	2 to 8	4 to16	633
Thr-86-Lys[106,138,199,203]	96 to 128		4 to >16	4							16
Thr-86-Ala[91,109,119,199]	32 to 256	4	0.094 to 2				0.25	1	0.25	0.5	6
Thr-86-Val[108]			4 to 64								3
Asp-90-Asn[105-109,119,121,137,138]	32 to 400	25 to 128	4 to 32	2 to 4	12.5		1 to 4	2 to 16	1 to 8	0.5 to 2	38
Asp-90-Tyr[105,138]	128		4 to 32								20
Asp-90-His[105]	64		4				1				1
Ala-70-Thr[121]	1		64								1
Ala-87-Pro[105]	64		2				0.5				1
Thr-86-Ile, Asp-90-Asn[105,107,137,200,201]	>128 to 512		2 to 256	64 to 128			16 to 32				0
Thr-86-Ile,Ser-22-Gly[120]	256		32								3
Thr-86-Ala, Ser-22-Gly[120]	256		0.19								1
Thr-86-Ile, Asn-203-Ser[122,132]	256		32 to128								10
Thr-86-Ile, Pro-104-Ser[108,118,201]	256		16								3
Thr-86-Ile, Ala-87-Pro[105]	>128		64				32				1
Thr-86-Ala, Asp-90-Asn[105]	128		64				16				1
Thr-86-Ile, Asp-90-His[105]	>128		128				16				1
Thr-86-Ile, Asp-85-Tyr[109]	>128	> 128	16				2	64	2	8	1
Asn-203-Ser, Ala-206-Thr[122]			64								1
Thr-86-Ile, Asn-203-Ser, Ala-206-Thr[122]			16								1
Thr-86-Ile, Asn-203-Ser, Ser-22-Gly[122]			16 to 64								7
Thr-86-Ile, Asn-203-Ser, Ser-22-Gly, Ala-206-Val[122]			32 to 64								3
Thr-86-Ile, Asn-203-Ser, Ser-22-Gly,Val-149-Ile[122]			16 to 64								22
None[106,108,109,113,117-119,121,132,137,200,201,204]	1 to 100	0.5 to 1	0.032 to 32	0.03 to 0.25	1.56	0.094	0.06	0.03 to 0.125	0.06 to 0.125	0.015 to 0.06	208
Total											**982**

(Continuation) Table 2.3
Sensitivity methodology was not taken into consideration but we avoid to report here any result of disk susceptibility. All results are reported in μg/ml or mg/L. Fluoroquinolones sensitivity results were recorded for: NAL (nalidixic acid), NOR (norfloxacin), CIP (ciprofloxacin), ENR (enrofloxacin), ORF (orfloxacin), LEV (levofloxacin), MOX (moxifloxacin), GRE (grepafloxacin), GAT (gatifloxacin), TRO (trovafloxacin).

CHAPTER 3

CHARACTERIZATION OF MUTATION FREQUENCIES, RECOVERED ANTIBIOGRAMS AND MUTANT PREVENTION CONCENTRAITON IN CAMPYLOBACTER JEJUNI STRAINS SUBSEQUENT TO A FLUOROQUINOLONE EXPOSURE [1]

[1]de Souza Amado, J. E., P. J. Fedorka-Cray, C. C. Brown, J. S. Bailey, and A. R. Woolums. To be submitted to Journal *of Antimicrobial Chemotherapy*.

Abstract

Antimicrobial resistance has become a global concern for public health since antibiotics were first introduced for clinical treatment of human and animal infections. In order for a new resistance mechanism to be discovered it appears to be just a matter of finding a new antimicrobial. One of the ways bacteria are capable of developing antimicrobial resistance is through spontaneous mutation.

The objective of this study was to examine the mutation frequency and the mutant prevention concentration (MPC) of strains (all from a single parent strain of *Campylobacter jejuni*) expressing various resistance mechanisms after exposure to four different fluoroquinolone antibiotics (nalidixic acid, norfloxacin, ciprofloxacin, enrofloxacin). The isolates included: a parent strain (81116); strain 81116 with an insertion deletion for the efflux pump *cmeB*, strain 81116 with an insertion deletion for the regulator gene of the efflux pump (*cmeR*), causing over-expression of the pump, and strain 81116 with a point mutation causing altered fluoroquinolone attachment to the *gyrA* subunit of gyrase rendering the strain resistant to fluoroquinolones.

Findings included equivalent mutation frequencies and MPCs between the parent strain and the strain with the enhanced efflux pump; and decreased mutation frequencies and MPCs in the strain with the decreased efflux pump activity. A significant difference occurred in the presence of colonies at 48 and 96h of incubation, indicating that a longer incubation period may be important in evaluating mutation frequencies. The recovery of survivors from concentrations at 96 hours after no growth was initially observed at 48 h prompted us to increase the MPC concentration of some isolates for that particular quinolone concentration.

Introduction

As reported by CDC, *Campylobacter* incidence in the U.S. in 2007 was 5,818 culturally confirmed cases, or a frequency of 12.79 per 100,000 Americans[1,2]. *Campylobacter* is recognized as one of the main causes of bacterial gastroenteritis throughout the world[2,3]. Case studies of sporadic gastroenteritis and genotype studies with human fecal samples and poultry products have linked human illness with *Campylobacter* to poultry meat consumption[4,5]. Many other retail foods[6] and also water[7,8] may be contaminated with *Campylobacter spp*. and unpasteurized milk has also been linked to outbreaks[7,9]. However, poultry products are thought to be responsible for the majority of sporadic campylobacteriosis cases [7].

Clinical symptoms including abdominal pain, headache and fever [10,11] are characteristic of campylobacteriosis and as well as other gastrointestinal diseases in humans. When antimicrobial therapy is prescribed, usually without culture confirmation, broad spectrum antibiotics, especially ciprofloxacin[12], are prescribed empirically. Ciprofloxacin is also a popular relatively non-specific treatment to prevent traveler's diarrhea[13] which results in many travelers taking this antibiotic when experiencing diarrhea during travel. In fact, consumption of ciprofloxacin to treat traveler's diarrhea has been cited as a risk factor for carriage of a fluoroquinolone resistant *Campylobacter*[14]. Furthermore, a correlation between harborage of fluoroquinolone resistant *Campylobacter jejuni* and a longer duration of clinical symptoms has been reported[14].

Commonly found in the intestinal track of commercial chickens and other fowl, *Campylobacter* is not a pathogen in birds but is considered as a commensal bacterium[15]. As a result, no therapeutic treatment is recommended for birds carrying *Campylobacter*. Nevertheless as part of the large population of gut flora, *Campylobacter* is exposed to antimicrobial treatments

aimed at infections such as colibacillosis. As a result, following exposure to drugs such as enrofloxacin, an antimicrobial treatment of choice for *Escherichia coli* air sacculitis infection in poultry, fluoroquinolone resistance emerged in “non-target” bacterial species such as *Campylobacter*[16-19].

Fluoroquinolone resistance in *Campylobacter* is mediated by at least two mechanisms. First, a gyrase gene mutation with subsequent modification of the structure on the gyrase subunit-A (*gyrA*) has been described. A point mutation in the *gyrA* gene decreases the affinity of the fluoroquinolone to this important gene replication protein. Second, an efflux pump, *CmeABC*, which functions constitutively to expel the antimicrobial from the cytoplasm, has also been described. When the regulator protein (*cmeR*) upstream of *CmeABC* is altered, there can be over-expression of the efflux pump resulting in enhanced resistance or expression of multiple antimicrobial resistance[20-23].

Single or coupled point mutations in the *gyrA* subunit coding sequence are linked by occurrence and have been described for *E. coli* point mutations that promote fluoroquinolone resistance[24]. These point mutations include: Thr-86-Ile[25-30], Thr-86-Ala[31-33], Thr-86-Lys[21], Asp-90-Asn[21,24,31,32], Ala-70-Thr[24], Pro-104-Ser[30,32,34], Ser-22-Gly, Asn-203-Ser, and Ala-206-Thr[35]. However, the only rigorously tested (by Molecular Koch’s postulate[36]) point mutation of the *Campylobacter gyrA* subunit was Thr-86-Ile [37].

It has also been established that the *CmeABC* tripartite efflux pump acts synergistically and constitutively with *gyrA* mutations to incrementally increase MIC results[21]. Another way that efflux pumps may influence antimicrobial resistance is by over-expression of *cmeABC*. *CmeABC* may be over-expressed after binding of the regulator protein *CmeR* to the promoter sequence is reduced. Modification of the intergenic region in the *cmeR*ABC operon between

cmeR and *cmeA* genetic sequences due to either a C-to-T transition[22] or a single nucleotide deletion[38] are a means of impairing the regulator protein.

Many investigators have demonstrated development of fluoroquinolone resistance in *Campylobacter jejuni* after broiler chickens are treated for *Escherichia coli* infection[16-19] or humans are treated with fluoroquinolones[39,40]. Developing a means to reduce or prevent the development of antimicrobial resistance would result in a clinical benefit. Recent investigations directed at understanding antimicrobial resistance mechanisms have focused on adjusting the antimicrobial dose and pharmacokinetic studies[41-43].

Mutant prevention concentration (MPC) is a relatively new strategy for studying antimicrobial potential by looking at a bacterium's relative capability to avoid antimicrobial resistance instead of only examining the therapeutic characteristics[44]. MPC is done by exposing approximately 10^{10}colony forming units (CFU) of a bacterial suspension to the antimicrobial of interest[44]. Minimum inhibitory concentration (MIC), the widely used tool to compare therapeutic usefulness of an antimicrobial against a microorganism, unlike MPC, is run with a lower count of 10^4 to 10^5 CFU[45-48]. The high MPC bacterial concentration is used to allow the measurement of the minimum antimicrobial concentration necessary to inhibit the development of a resistant sub-population of an isolate[44]. The mutant selection window (MSW) is defined as the antimicrobial concentrations that are between the measured MIC and the MPC[44]. It is at the MSW that resistant sub-populations of an isolate will be propagated.

Mutation frequencies for fluoroquinolone resistance in *Campylobacter* have been determined for various strains by several investigators[49-51]. However, to date there has not been a meaningful explanation regarding interactions of these mutation frequencies with drug concentrations or any description of observed antimicrobial resistance profiles. In this study, the

effects of the two main mechanisms of *Campylobacter* resistance on mutation frequencies were examined through the use of genetically modified strains of bacteria[51] by determining the MPC of four different fluoroquinolones against four genetically altered strains of *Campylobacter*[52].

Materials and Methods

Bacterial strains and culture methodology

Bacterial strains used are listed in Table 3.1. A parent strain, *Campylobacter jejuni* 81116[8,53,54] , fully susceptible to fluoroquinolones, was the prototype (referred to as *P* for Parent). The other three strains were genetic modifications of this strain. Genomic DNA, carrying *cmeB* (referred to as -*B*)[55] and *cmeR* (referred to as -*R*)[38] insertionally deleted within kanamycin and chloramphenicol resistant cassettes, respectively were provided by Dr Qijing Zhang (Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA)[51]. *Campylobacter jejuni* 81116[8,53,54], a stable and genetically sequenced strain first isolated from humans, was naturally transformed with the DNA as described above using the biphasic methodology[56]. Guidance for the first natural transformations was also kindly provided by Dr Q. Zhang and his research group. In addition, an 81116 ciprofloxacin resistant strain (identified as *C*) was chosen from one of the trials where the parent 81116 was exposed to 4µg/ml of ciprofloxacin for 48h. This strain presented a Thr-86-Ile mutation at the quinolone resistance determining region (QRDR) of the *gyrA* gene as determined by sequencing.

Transformants carrying insertional deletions of the genes *cmeB* (-*B*) and *cmeR* (-*R*) were grown on Mueller Hinton agar (MHa) containing 30µg/ml of kanamycin and 8µg/ml of chloramphenicol, respectively. All strains were kept in modified Wang's[57] freezing media in well sealed vials stored at -80°C. Copies of the same strain were kept frozen and tested for their

antibiogram profiles[48,58] prior to experimental procedures to ensure that the genotypic and phenotypic characteristics were maintained.

Unless stated otherwise *Campylobacter* isolates were grown at 42°C for 24-48h to ensure adequate growth. Microaerophilic conditions were maintained by introducing the inoculated media into a one gallon zip-lock bag saturated with an air mixture of 10% CO_2, 5% O_2 and 85% N_2[59].

Microbiological media:

Microbiological culture media was prepared according to manufacturer's instructions. Plain Mueller Hinton agar and broth (MHa and MHb, respectively ; Hardy Diagnostics, Santa Maria, CA) were used as non-selective media for culture propagation. Mueller Hinton blood agar (MHBa) plates were prepared by adding 5 % sheep defibrinated blood (Hema Resource & Supply, Oregon). Campy-Cefex agar (CCa) plates were prepared as described[60]. The CCa plates were used to ensure isolate purity. Bolton broth[61] (BB) was prepared without the use of antimicrobials as an enrichment broth. *Campylobacter* enrichment broth (Accumedia, Baltimore, MD) and 5% (vol/vol) lysed horse blood (Lampire Biologicals, Pipersville, PA) were used to make non-selective BB. Brucella broth (Sigma-Aldrich, St Louis, MO) was used for the Wang's media and included the addition of 15% (vol/vol) glycerol[62] (J.T. Baker, Phillipsburg, NJ) and 10%(vol/vol) lysed horse blood prior to use. Ready-to-use blood agar plates (Remel, Lenexa, Kansas) were used as a non-selective culture medium.

Antibiotic containing media was made for the relative mutation frequency trials, for the agar dilution methodology, and to select and cultivate the transformants (products of natural transformation). All antibiotic containing media were prepared according to CLSI guidelines[48,58],

drug manufacturer's instructions, or taking into consideration literature-derived evidence of drug stability under storage[46,63]. Stock solutions of 10,240µg/ml to 20,480µg/ml were prepared with the appropriate solvents and diluents according to CLSI[58] and stored at -80°C for no more than 6 months. Antimicrobials known to have a history of instability after storage at freezing temperatures (tetracycline and ampicillin) were prepared and used for making media on the same day[46,63] or stored overnight at 4°C. Once thawed, antimicrobial stock vials were either used to completion or discarded the same day. Tetracycline hydrochloride, nalidixic acid salt, erythromycin, and chloramphenicol were purchased from Sigma-Aldrich (St Louis, MO). Ciprofloxacin hydrochloride, norfloxacin hydrochloride, enrofloxacin and ampicillin anhydrous were acquired from MP Biomedicals (Solon, OH). Kanamycin sulfate was purchased from USB (Cleveland, OH). All antimicrobials were stored according to the manufacturer's directions.

Plates containing antimicrobials were prepared by incorporating appropriate amounts of the stock solutions according to CLSI recommendations[48] into MHa plus 5% (vol/vol) defibrinated sheep blood immediately prior to pouring. Selective plates used for cultivation of kanamycin and chloramphenicol resistance cassettes carrying transformants, were the only antibiotic containing plates without sheep blood. Antibiotic containing plates were used within 7 days or discarded. All microbiological media were refrigerated at 4°C, protected from light, and used within the time frame suggested by the manufacturers. Quality control was conducted on one randomly selected agar plate at each antimicrobial concentration for each batch of media using ATCC33560 (*C. jejuni*) and ATCC25922 (*Escherichia coli*)[48].

Relative mutation frequency determination and the mutant prevention protocol

All bacterial strains (*P*, *C*, *-B* and *–R)* from the -80°C freezer were inoculated onto three MHa plates. After incubation at 42°C for 24 h as described, single colonies were transferred to new plates to ensure hardiness and minimize non-viable cells; plates were incubated again under the same conditions. For each strain six 1µl loops were taken from the areas of lighter growth on the plates and inoculated into 30ml of MHb in a 50ml Falcon tube. Optical density of the cultures was measured at 600nm (OD_{600}) on a SpectraMax® Plus*384* (MDS Analytical Technologies, Toronto, Canada) spectrophotometer. Tubes were incubated for 24h under microaerophilic conditions with partially unscrewed lids at 42°C. After 24hs, optical density was determined and adjusted to approximately 0.08 ± 0.01 $OD_{600.}$ The suspension was diluted 1:100 in multiple tissue culture flasks with vent caps (Sigma-Aldrich, St Louis, MO) containing approximately 500ml for 24hs. After 24h the cultures were centrifuged at 3,000 rpm for 30mins at 4°C and the pellets were resuspended in MHb to an OD_{600} of 1.0 to 3.5. Approximately 300 to 350µl of the suspension was spread on antimicrobial plates with increasing log2 concentrations of either nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP), and enrofloxacin (ENR). The inoculated plates were air dried and incubated at 42°C under microaerophilic environment for a maximum of 96 hours. The goal was to expose a total of 10^{10} CFUs (over 11-15 plates) per antimicrobial concentration. Plate counts on MHBa plates were done to ensure the target CFUs were reached.

Plates were examined at 48 and then at 96 hours in order to observe the appearance of any additional colonies. Groups of plates (divided by concentration) presenting full growth, were recorded as 100% of growth. Therefore, when the relative mutation frequency was recorded for statistical analysis it was entered as one (result of the ratio of number of exposed cells to the

number of cells that grew) or zero after log10 transformation. The ratio was necessary to allow statistical analysis to be calculated with the confluent growth isolates. Groups of plates with no growth were recorded as the limit of detection. The same groups of plates with no growth were recorded as MPC48 or MPC96 according to the sampling time. Groups that presented isolated growth had their total number of colonies recorded at both 48 and 96 h for that particular antimicrobial concentration. Relative mutation frequencies of isolated colonies were calculated by dividing the total number of mutants by the total number of cells exposed. Experiments were conducted twice and results were kept separate for the purpose of statistical analysis. Five isolated colonies were picked from each concentration, at each sampling time, from one replicate plate per drug.

Mutant Prevention Protocol Survivors Enrichment

Five milliliters of Bolton broth were aseptically poured onto all plates with no visible growth at 96h. A sterile hockey-stick was used per plate to suspend any possible bacteria and a sterile pipette was used to remove the broth and place it in individual broth tubes that were incubated for 48h. After incubation, each tube was vortexed and 100µl was plated onto MHa plates that were incubated for 48h as described. Any visible growth was observed by wet mount under phase contrast to ensure conformity with *Campylobacter* morphology[59]. Presumptive positive plates for *Campylobacter* were passed to a BA plate, incubated for 24hs then frozen at -80°C in Wang's modified freezing media for further susceptibility testing.

Antimicrobial susceptibility testing

Both agar replica plate and agar dilution methodologies were used. Replica-plate methodology was adapted to make it relevant for *Campylobacter*[64]. In this study the primary plates were MHBa. Isolates were replicated onto a non-selective plate before replication onto antimicrobial plates containing the same drug and concentration they were exposed to. The MHBa plates were replicated as controls before and after the antimicrobial plates of interest. Plates were always grown for 24h as described.

Agar dilution testing was performed on isolates before exposure to the various fluoroquinolones. The minimal inhibitory concentrations (MICs) of tetracycline (TET), ampicillin (AMP), erythromycin (ERY), kanamycin (KAN), chloramphenicol (CHL), nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP) and enrofloxacin (ENR) were determined by the agar dilution method recommended by the CLSI[48]. Isolates were exposed to doubling concentrations of each antimicrobial using a Steer's replicator. Two MHBa plates were used as positive controls before and between each drug tested. Tests were run at least 2 times for a majority of the isolates. MICs were reported as averages after a log2 transformation of the concentrations. ATCC33560 and ATCC25922 of *Campylobacter jejuni* and *Escherichia coli,* respectively were used as controls following CLSI protocol[48]. Strains 81116 and its isogenic mutants were also used as controls to asses both the stability of the antibiogram of the isolates and the protocol. Table 3.1 contains the mode results of antimicrobial sensitivity tests run for the controls.

DNA sequencing

Colonies were randomly selected from the isolates that underwent susceptibility testing. DNA was extracted using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to manufacturer's directions. Primers GZgyrA5 (ATT TTT AGC AAA GAT TCT GAT) and GZgyrA6 (CCA TAA ATT ATT CCA CCT GT)[30] were used to amplify the QRDR of *gyrA* (210bp) present in a 673bp product. Amplification protocol consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles of 94, 50 and 72°C for 1min each and a final extension at 72°C for 10min. The PCR reaction consisted of a 50µl solution containing the following: 1µl of DNA, 5µl of 10x PCR buffer, 1µl of 10mM dNTP's, 0.5µl of each primer (giving a final concentration of 50pM for each), 0.25µl of a 1.25U/reaction Taq(Quiagen) and DNase free water to complete the reaction volume. The PCR products were purified using a QIAquick® PCR purification Kit (QIAgen, USA). Reverse and forward sequencing reactions were performed using the same primers of the PCR reaction. Reactions were performed using an Applied Biosystem 3730 DNA sequencer (performed by Dr David S. Needleman, USDA, ARS, ERRC, Integrated Biomolecular Resources, Wyndmoor, PA). Data was assembled and compared using Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, MI).

Statistical Analysis

Analysis was done with using SAS (version 9; SAS Institute, Cary, NC). Concentrations were transformed into log2 for averaging and statistical analysis while relative mutation frequencies were log10 transformed. The paired Student T test and ANOVAS were run to check for significant statistical differences.

Results

Relative Mutation Frequency detection

An increase in the number of visible colonies was observed from 48h to 96h at almost every combination of drug and isolate. An exception was *C* which when exposed to NAL, had no increase in the number of colonies between 48 and 96h. This trend was common on both replicates. A paired T-test comparing the log10 transformed mutation frequencies recorded at both times for all fifteen concentrations of the four different fluoroquinolones and four different isolate combinations, taking the two replications in consideration (n=480), indicated that sampling time difference was statistically significant ($p<0.001$).

Tables 3.2 through 3.9 display the relative mutation frequencies for all of the isolates subjected to the four different antibiotics at both 48h and 96h versus selected concentrations. In general for the *C* isolate there was confluent growth at both 48h and 96h for all drugs. The *P* and *-R* strains were similar to each other, and different from the *C* isolate values at every concentration and both time points. At most concentrations and both time points, *-B* remained the same in comparison to *P* and *-R*. However, at the highest concentrations, strain B was different ($p<0.05$) from the *P* and *-R* strains and exhibited a lower mutation rate.

Relative mutation frequencies calculated from colony numbers at 48h and 96h of each experiment were submitted to two different analyses of variance. In sum , 4 isolates were exposed to 4 different fluoroquinolones at 15 different concentrations with 2 replicates (0.03125µg/ml in log2 increments until 512µg/ml) resulting in 120 pooled samples by isolate or by drug for each sampling time. Our objective was to compare the pool of all relative frequencies and determine the influence of isolates, antimicrobials, or their concentrations at each sampling time point. Data indicate that all three factors had a significant ($p<0.001$) effect on the means at

both 48h and 96h. Using Tukey's test a comparison of the means from each isolate (n=120 for each mean) of the pooled data of all exposures indicated that isolate *C* as the highest relative mutation frequency mean followed by -*R* and *P* which exhibited equal means followed by -*B* which exhibited the smallest mean. These results indicate not only an influence of the relative mutation frequencies, but of the concentrations that were below the MIC results of each isolate. Isolate *C*, which exhibited high resistance, showed a high relative mutation frequency. The opposite was observed for -*B*.

<u>The MPC protocol:</u>

Growth was classified as confluent, isolated, or no growth. Confluent growth was seen at lower concentrations closer to the original MIC (MIC_{orig}) of each isolate for all fluoroquinolones. Isolated growth was generally seen one to two concentrations above the MIC_{orig} and therefore inside the mutant selection window (MSW) of each isolate. The only strain exposed to fluoroquinolones that had no isolated growth was -*B* exposed to NAL. Plates with no growth were found between one to eight concentrations above the MIC_{orig}, and were called MPC48h or MPC96h. The MPC for plates exhibiting no growth at 96h were always enriched to check for survivors. Whenever survivors were found, the MPC96h was corrected to reflect such a recovery and noted as MPC_{correc}.

Results for MPC are presented in Table 3.10. To further confirm the MPC48h and 96h differences as significant, transformation by log2 of the MPC results (concentrations) was done and the influence of sampling time was checked by paired Student-T test on the pooled results of isolates versus drugs in two independent replicates (n=32). The difference between MPC48h and MPC96h was considered statistically significant at $p<0.0001$.

To better understand the influence of efflux pumps on the MPC the 48 and the 96h MPC results were subjected separately to ANOVA testing followed by Tukey's honest test on an n=32 composed of 4 different isolates versus 4 different fluoroquinolones in replication. Results indicated that isolate ($p<0.0001$), antimicrobials used as challenge ($p<0.0001$), and interaction of both ($p=0.0173$) were influential factors on pooled means of the isolates and also of the different fluoroquinolones. Tukey grouping of the isolate comparison indicated that the highest log2 pooled mean was *C* (6.75) followed by -*R* and *P* (respectively 4.75 and 4.375) with the lowest belonging to -*B* (3.375). Such result confirms the observations in Table 4.3 that -*B* is clearly 2 fold lower than *P* and -*R*.

The recovered MIC (MIC_{rec}) was consistently higher than the MIC_{orig} and was consistent across all strains and all four fluoroquinolones. The MIC_{rec} for *C,* although much higher than isolate *P* MIC_{orig} was not much higher than its' own MIC_{orig}, except when CIP or ENR was the antimicrobial.

To assess the influence of efflux pumps on the MPC, two different analyses of variance were run with the MPC results of both 48h and 96h protocols. An n=8 per isolate and per drug was run after a log2 transformation of the MPC results. Isolate and antimicrobial were significant factors on the process ($p<0.001$). Using Tukey's these data indicate that -*B* is the most vulnerable isolate to the MPC of fluoroquinolones, followed by P and -R together, while isolate C is the most resistant of them at both sampling times. The same Tukey comparison was made to evaluate both 48 and 96h means for the fluoroquinolones and show statistically which antimicrobial is the most and least effective in killing the four isolates. The result organized from the highest MPC to the smallest concentration is NAL, followed by NOR for both 48 and 96h.

However beginning at 48h CIP and ENR have similar means. At 96h CIP has a higher mean than ENR.

Antimicrobial susceptibility testing and DNA sequencing

A total of at least 35 susceptibility tests were run with unexposed isolates *P*, *C*, *-B* and *-R* and these results were used as a control to test the stability of susceptibility testing with *P* serving as the reference isolate. Susceptibility results for isolate *-B* show a resistance decrease of 2-fold (2X) to CHL, NAL, NOR and ENR, 4X decrease to TET and CIP, 8X decrease to ERY and 64X decrease to AMP when compared to its parent strain *P*. In its place *-R* presented almost no change in its antibiogram when compared to the parent strain. Isolate *C*, which was selected after *in vitro* exposure to CIP presented a 32X increase of NAL, 256X NOR, 128X CIP and a 64X ENR when mode results were compared to parent strain P (Table 3.1).

Isolates from each of the replica plates were tested and results are recorded in Table 3.10 at the MIC_{rec} columns (only fluoroquinolone results of MIC_{rec} are shown since all the other antimicrobials (TET, KAN, AMP, ERY, CHL) had no appreciable change). MIC_{rec} results were always greater or equal to the fluoroquinolone concentration plate value they came from. This observation holds true for both replica-plating and agar dilution.

Sequencing of the *gyrA* was performed in 22 selected strains from the isolated colonies and survivors listed in Table 3.11. A comparison of the antibiograms of sequenced strains versus not sequenced strains is shown in Table 3.12. The majority of the colonies (73%) exhibited the Thr-86-Ile substitution. Other sequences predicted and their observed percentages were Thr-86-Ala 15%, Asp-90-Gly at 6%. From these findings 15% would be expected as having no

mutation while being NAL resistant (32 to 128μg/ml), suggesting that there is some other mechanism of NAL resistance besides the QRDR mutations in the gyrA.

A rare QRDR substitution (Asp-90-Gly) was observed in an isolate presenting a Nal only resistant profile at 256μg/ml. This point mutation was found in two different isolates: a *P* strain selected from an ENR challenge of 0.25μg/ml and an *R* isolate selected from a NOR challenge at 2μg/ml; both colonies were selected at 96hs (table 3.11)

Discussion

This work was designed to test the contributions of two resistance mechanisms on the mutation frequencies and MPC results of four different isogenic isolates. The use of 81116, a genetically sequenced and known strain allowed better control during testing which increased reproducibility. The strain that was insertionally deleted for the *cmeR* causing over-expression of the efflux pump proteins and enhanced extrusion of antibiotics from the bacterial cell, performed in all cases similarly to the parent strain in terms of mutation frequency. Another strain, -*B*, was insertionally deleted for the efflux pump *cmeB* protein resulting in no extrusion of antibiotic from the cytoplasm. In our results, there was a clear indication that strain -*B* had a reduced tendency to mutate when exposed to high concentrations of the test antibiotics. However, this tendency was not as high and clear as the one recorded by Yan *et al* 2006[51] although in this study ciprofloxacin was used at only three different concentrations, 8μg/ml and lower, and results were recorded at 48h only. At the lowest concentration (0.0625μg/ml), all three strains showed the same mutation frequencies; as concentration increased to 4μg/ml, the isolate with *cmeB* decreased in mutation frequency in comparison to the others, which is similar to our finding. However, *cmeR* in Yan *et al* [51] demonstrated a higher mutation frequency as the

concentration of fluoroquinolones increased which confirms over-expression of the efflux pump[51].

With the *cmeB* insertional deletion, more drug is likely to enter within the cell. At the highest concentrations, there would be considerable amounts of antibiotic within the cell, so the bacteria would be expected to die earlier, which could explain the lower mutation frequency[51]. To simply classify a bacterium as hypomutable or hypermutable without knowing its MIC could be considered inaccurate. In a recent article *C. jejuni* and *C. coli* strains were exposed to 1ug/ml of ciprofloxacin[50]. Relative mutation frequencies were recorded as ranging from 4×10^{-9} to 7×10^{-3}, hypomutable to hypermutable, respectively[50]. The results of our study do not demonstrate such a wide range of mutation frequency, perhaps because we only used one strain and its variants. Additionally, the strongly mutable isolates were also resistant to TET 32μg/ml or AMP 16 μg/ml[50]. In our study, none of the isolated organisms demonstrated resistance to antibiotics other than the fluoroquinolones.

An unexpected result was that there was a statistically significant difference in the number of colonies (and therefore mutation frequencies) when the challenge plates were incubated for an extra 48 hours. Because fluoroquinolone therapy in humans or animals generally occurs for more than 2 days (>48h), the *in vitro* data at 48h may have insufficient clinical relevance, and actually undervalue the possibility of further mutations.

Five colonies were randomly picked from every concentration that presented isolated growth in all exposures of all 4 variants at both sampling times. These five colonies were submitted to replica plating before freezer storage and to agar dilution after frozen. Both the replica plating results and the agar dilution results shows that the resistance profile was the same for colonies at either 48h or 96h, with all picked colonies being at least resistant to the

concentration and fluoroquinolone equivalent to the plate they were selected from. The fact that all isolates tested with the replica-plate methodology were considered resistant to the concentrations and drugs they were exposed to confirms the stability of the fluoroquinolone concentration in the incubated plates. This is especially important for the isolates picked at 96h. It could be argued that these 96h isolates were strains that were altered in some capacity at that particular antimicrobial concentration to grow well. However, because the resistance profiles were the same as those at 48h, it suggests that the 96h resistant colonies are altered in some way not related to resistance. For instance these late bloomers may be injured cells that despite their resistance just had a lower growth rate.

The fact that the MIC_{rec}. for *C,* although much higher than isolate *P* MIC_{orig} was not much higher than its own MIC_{orig}, except when CIP or ENR was the antimicrobial leads us to conclude that after a Thr-86-Ile mutation there are not many mutations of the *gyrA* capable of increasing resistance against NAL or NOR. This is corroborated by the fact that Table 3.11 shows the sequencing results for *C* isolates and despite their increase in MIC onlyThr-86-Ile mutations are found with the exception of the one with the Asp-90-Asn mutation.

Our MPC results consistently show (with 2 independent replicates for each fluoroquinolone) that a *cmeB* insertional deleted mutant (-B) presented a 2- to 4-fold decrease of all the MPCs recorded (48h, 96h and corrected). This is a clear indication that the efflux pump has an effect on MPC in that inhibition of the pump's activity decreases the MPC. Consequently, lower therapeutic doses are necessary to prevent emergence of antibiotic resistant strains if the efflux pump has already been compromised. However, no strain with a damaged efflux pump has yet been isolated, which is probably due to use of selective antimicrobial plates such as Cefex and CVA. Strain *-B* could never grow in either one of the commonly used

selective plates. Development of drugs that specifically target the efflux pump to inactivate it could be accompanied by use of fluoroquinolones at lower dosages.

The first published article to examine MPCs of *Campylobacter jejuni* explored the MPCs of two fluoroquinolone-susceptible strains against CIP, ENR and NAL. In their discussion they compared the MPC results to the literature available maximum serum concentrations (C_{max}) of CIP in humans (C_{max} =4.4µg/ml in a dose of 750mg/day)[65] and of ENR in chickens (C_{max} =2.44µg/ml after a dose of 10mg/kg)[66]. Most of the fluoroquinolones' MPCs for this study were higher than the C_{max} at normal dosages in humans and chickens .Only the *-B* isolate's MPC 48 and 96h with their 2µg/ml result were below the C_{max} reported for chicken when the *-B* was exposed to ENR. Therefore since *cmeB* deletion brought the strain MPC to a level much closer to the C_{max} this suggests that if we find a way to harness this characteristics we could make a wild-type *Campylobacter* more susceptible to the ENR MPC by simply administrating an efflux pump inhibitor along with it. However, it is important to remember that fluoroquinolones are a class of drugs with a concentration-dependent bactericidal effect. Therefore an area under the 24h time-concentration curve (AUC_{24}) divided by the MPC would be the best indicator for the effectiveness of this drug class[44]. Paradoxically, for both this study and the previously published by Pasquali *et al* [52] for ENR in chickens, it was the fluoroquinolone that mostly approximated of a C_{max} of normal chicken dosages. This result is somewhat ironical since as a result of the development of antimicrobial resistance in *Campylobacter* ENR approval was withdrawn by the FDA[67], while at the same time CIP is largely used to treat non-culture confirmed cases of diarrhea in humans.

Standard measurements for *Campylobacter jejuni* growth vary from 24h to 48h depending on the protocol. Fluoroquinolones are bacteriostatic at most concentrations, but

considered bactericidal at higher concentrations[20]. The fluoroquinolone's bacteriostatic effect could decrease the growth rate of *Campylobacter*. For previous MPC experiments, most readings are done at 24h and 48h. However, because of the concern about growth rate, for this study we examined MPC at both 48h and 96h. The enrichment of possible survivors of the so called MPC96h plates brings a further stringency to the MPC results. The fact that the survivors were resistant to the fluoroquinolone concentration against which they were exposed to is a great signal that although invisible to the naked eye, they are a probable risk and need to be accounted for when using MPC methodology.

In summary the findings from this study suggest that MPC96h should be the sampling time of choice when assessing the concentrations necessary to prevent mutant enrichment in *Campylobacter jejuni* and its relative mutation frequency. It is suggested that enrichment of the first concentration with no growth should become a normal procedure to allow even the smallest resistant colonies to be assessed. In addition, it could be speculated that if an effective and safe efflux-pump inhibitor could be found, it would be an invaluable asset to boost the mutant prevention capability of the fluoroquinolones. Additionally, fluoroquinolones should not be used by themselves for the treatment of *Campylobacter jejuni* in humans under the auspices of the MPC methodology.

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Table 3.1 Strains of bacterial isolates and isogenic mutants used in this study

	Description	Used as[j]	*MIC (µg/mL)								
			AMP[a]	TET[b]	CHL[c]	KAN[d]	ERY[e]	NAL[f]	NOR[g]	CIP[h]	ENR[i]
ATCC33560	C. jejuni	S and R	4	1	2	16	1	8	0.25	0.125	0.0625
ATCC25922	E. coli	S and R	8	1	4	4	128	4	0.125	0.015625	0.03125
P	C. jejuni 81116 wild-type	S and E	2	0.25	2	8	0.5	8	0.25	0.125	0.0625
-B	C. jejuni 81116 derivative *cmeB::kan*	S and E	0.03125(-64)	0.0625(-4)	1(-2)	512(64)	0.0625(-8)	4(-2)	0.125(-2)	0.03125(-4)	0.03125(-2)
-R	C. jejuni 81116 derivative *cmeR::cm*	S and E	1(-2)	0.25	16(8)	8	0.5	8	0.25	0.125	0.0625
C	C. jejuni 81116 derivative *gyrA* substitution Thr-86-Ile	S and E	2	0.5(2)	2	16(2)	0.5	256(32)	64(256)	16(128)	4(64)

P=parent strain, *-B*=*cmeB* insertionally deleted variant, *-R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant
* Results grouped as MIC are referent to mode concentrations found for each isolate on agar dilution testing of each antimicrobial: [a]Ampicillin, [b]Tetracycline, [c]Chloramphenicol, [d]Kanamycin, [e]Erythromycin, [f]Nalidixic acid, [g]Norfloxacin, [h]Ciprofloxacin, [i]Enrofloxacin.
[j]Comprehend usage information. The strain could have been used as: a standard in susceptibility testing (S), a strain to be exposed at different treatments (E) or a replica plate standard (R).

Table 3.2 Relative mutation frequencies of 81116 and its variants at 48h after exposure to ciprofloxacin

CIP 48h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
3	8	**<-10.46±0.11**[b]	CG[a]	**<-10.33±0.44**[b]	**-8.58±1.77**[b]
2	4	**<-10.46±0.11**[c]	CG[a]	-7.46±0.07[b]	-7.41±0.25[b]
1	2	-7.77±0.72[b]	CG[a]	-7.48±0.06[b]	-7.26±0.28[b]
0	1	-7.44±0.35[b]	CG[a]	-7.31±0.007[b]	-7.14±0.32[b]
-1	0.5	-7.30±0.36[a]	CG[a]	-3.59±5.07[a]	-3.61±5.11[a]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.3 Relative mutation frequencies of 81116 and its variants at 96h after exposure to ciprofloxacin

CIP 96h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
3	8	**<-10.46±0.11**[b]	CG[a]	-9.04±0.97[b]	-8.00±1.11[b]
2	4	**<-10.46±0.11**[c]	CG[a]	-7.46±0.07[b]	-7.34±0.16[b]
1	2	-7.56±0.46[b]	CG[a]	-7.41±0.01[b]	-7.20±0.23[b]
0	1	-7.40±0.32[b]	CG[a]	-7.26±0.04[b]	-7.11±0.04[b]
-1	0.5	-7.28±0.36[a]	CG[a]	-3.57±5.05[a]	-3.59±5.08[a]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.4 Relative mutation frequencies of 81116 and its variants at 48h after exposure to enrofloxacin

ENR 48h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
2	4	**<-10.23±0.33**[c]	CG[a]	**<-10.17±0.22**[c]	-7.47±0.41[b]
1	2	**<-10.23±0.33**[c]	CG[a]	-7.28±0.62[b]	-7.38±0.46[b]
0	1	-7.19±0.16[b]	CG[a]	-6.97±0.30[b]	-7.22±0.46[b]
-1	0.5	-7.16±0.23[b]	CG[a]	-6.89±0.37[b]	-7.31±0.45[b]
-2	0.25	-7.06±0.18[b]	CG[a]	-6.82±0.33[b]	-6.81±0.02[b]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth.
P=parent strain, *-B*=*cmeB* insertionally deleted variant, *-R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.5 Relative mutation frequencies of 81116 and its variants at 96h after exposure to enrofloxacin

ENR 96h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
2	4	**<-10.23±0.33**[c]	CG[a]	-8.07±0.51[b]	-7.43±0.39[b]
1	2	**<-10.23±0.32**[c]	CG[a]	-7.27±0.62[b]	-7.34±0.44[b]
0	1	-7.18±0.15[b]	CG[a]	-6.97±0.30[b]	-7.21±0.46[b]
-1	0.5	-7.14±0.22[b]	CG[a]	-6.88±0.36[b]	-7.27±0.46[b]
-2	0.25	-7.05±0.18[b]	CG[a]	-6.81±0.33[b]	-6.72±0.10[b]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth.
P=parent strain, *-B*=*cmeB* insertionally deleted variant, *-R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.6 Relative mutation frequencies of 81116 and its variants at 48h after exposure to nalidixic acid

NAL 48h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
8	256	**<-10.23±0.42**[b]	CG[a]	**<-10.61±0.06**[b]	**-9.98±0.21**[b]
7	128	**<-10.23±0.42**[b]	CG[a]	**-8.94±2.36**[b]	**-8.45±2.05**[b]
6	64	**-8.79±1.60**[b]	CG[a]	-7.37±0.21[b]	-7.25±0.44[b]
5	32	-6.91±0.21[a]	CG[a]	-3.66±5.17[a]	-7.10±0.49[a]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.7 Relative mutation frequencies of 81116 and its variants at 96h after exposure to nalidixic acid

NAL 96h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
8	256	**<-10.23±0.43**[b]	CG[a]	**-9.04±2.28**[b]	**-8.66±1.66**[b]
7	128	**-7.92±0.88**[b]	CG[a]	-7.74±0.70[b]	-7.56±0.83[b]
6	64	**-7.22±0.21**[b]	CG[a]	-7.34±0.17[b]	-7.12±0.27[b]
5	32	-6.81±0.29[a]	CG[a]	-3.64±5.16[a]	-6.99±0.35[a]

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant, CG=confluent growth.

Table 3.8 Relative mutation frequencies of 81116 and variants at 48h after exposure to norfloxacin

NOR 48h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-R (log10)
5	32	**<-10.23±0.33**^{b}	CG^{a}	**<-10.17±0.22**^{b}	**<-10.10±0.54**^{b}
4	16	**<-10.23±0.33**^{c}	CG^{a}	-7.18±0.48^{b}	-7.54±0.17^{b}
3	8	-7.38±0.34^{b}	CG^{a}	-7.12±0.46^{b}	-7.38±0.33^{b}
2	4	-7.28±0.21^{b}	CG^{a}	-6.83±0.20^{b}	-7.25±0.36^{b}

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant

Table 3.9 Relative mutation frequencies of 81116 and its variants at 96h after exposure to norfloxacin

NOR 96h					
		Isolates			
Log2 Conc	Conc.(μg/ml)	-B (log10)	C (log10)	P (log10)	-*R* (log10)
5	32	**<-10.23±0.33**^{c}	CG^{a}	-7.39±0.45^{b}	-7.45±0.42^{b}
4	16	-7.76±0.51^{b}	CG^{a}	-7.16±0.46^{b}	-7.44±0.27^{b}
3	8	-7.28±0.23^{b}	CG^{a}	-7.11±0.46^{b}	-7.37±0.34^{b}
2	4	-7.24±0.36^{b}	CG^{a}	-6.83±0.19^{b}	-7.24±0.36^{b}

Mean ± standard deviations after log10 transformation of two independent experiments. Different letters at the same row differ significantly (P<0.05, ANOVA). Results in bold correlate to means that present at least one of the replications showing no growth. *P*=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant

Table 3.10 Mutant prevention protocol: result summary table

[a]FQ exposure	Isolate	[b]MIC_{orig} (μg/ml)	[c]MIC_{rec} (μg/ml)				[d]MPC48h (μg/ml)	[e]MPC96h (μg/ml)	[f]Survivors (μg/ml)	[g]MPC_{correc} (μg/ml)
			NAL	NORF	CIP	ENR				
NAL	P	8	32 to 384	0.75 to 64	0.25 to 16	0.25 to 6	128 & 256	256 & 512		512
	B	4	ND	ND	ND	ND	64 & 128	256		256
	R	8	48 to 512	1 to 96	0.375 to 16	0.25 to 8	128 & 256	512		512
	C	256	256 to 512	64 to 128	16 to 24	6 to 8	512	512	512	512 & 1024
NOR	P	0.25	8 to 384	0.625 to 64	0.156 to 16	0.156 to 4	32	64		64
	B	0.125	4 to 256	0.125 to 32	0.03125 to 12	0.03125 to 10.67	16	32		32
	R	0.25	8 to 256	0.75 to 64	0.18 to 16	0.093 to 10	32	64		64
	C	64	192 to 256	96 to 192	12 to 16	4 to 8	64 & 128	128	128 & 128	256
CIP	P	0.125	8 to 512	1.5 to 64	0.5 to 16	0.25 to 6.7	8	8 & 16	8	16
	B	0.03125	192	24 to 32	4	2	4	4		4
	R	0.125	128 to 512	4 to 128	2 to 24	1 to 8	4 & 8	16		16
	C	8	256	64 to 128	48 to 80	8 to 128	64	64	64	128
ENR	P	0.0625	32 to 256	2 to 128	0.5 to 16	0.25 to 8	4	8		8
	B	0.03125	10 to 192	0.25 to 40	0.09375 to 6	0.0625 to 9	2	2	2 & 2	4
	R	0.0625	16 to 298.67	1.42 to 96	0.5 to 16	0.5 to 8	8	8		8
	C	4	192 to 256	64 to 128	32 to 64	32 to 128	64	128		128

P=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant
[a]Fluoroquinolone exposure column.
[b]Mode results of original minimal inhibitory concentration(MIC_{orig}) for each isolate versus the fluoroquinolone exposure.
[c]Range of minimal inhibitory concentration results of isolates recovered (MIC_{rec}) from various concentrations of the specific fluoroquinolone exposure.
[d]Mutant prevention concentration checked after 48h of incubation.
[e]Mutant prevention concentration checked after 96h of incubation.
[f]Concentration from where survivors were enriched out of the plates that showed no growth at 96h.
[g]Mutant prevention concentration 96h corrected for survivors.

Table 3.11 Select isolates: sequencing results and antibiogram profiles

Isolate	Sampling	[a]Drug & conc.(ug/ml)	Sequencing Result	[b]Effect	Nal MIC	Norf MIC	Cip MIC	Enr MIC
[d]*P*(81116)	-	-	-	-	8	0.25	0.125	0.0625
Recovered *P* colonies								
*P*3-2-4	48h	ENR at 2	Thr-86-Ile	Replacement	[c] **256**	**64**	**16**	**4**
*P*3-21-4	96h	ENR at 0.25	Asp-90-gly	Replacement	**256**	2	1	0.5
*P*3-3-2	48h	NOR at 16	Thr-86-Ile	Replacement	**256**	**64**	**16**	**4**
*P*3-26-2	96h	NOR at 1	-	Synonym	**16 to 128**	1 to 2	0.25 to 2	0.125 to 1
*P*1-22-3	96h	CIP at 0.25	-	-	**8 to 128**	2	0.5	0.25
*P*1-1-1	48h	NAL at 64	Thr-86-Ile	Replacement	**256 to 512**	**64**	**16**	**4 to 8**
*P*1-15-1	96h	NAL at 32	-	-	**32**	0.5 to 1	0.25 to 0.5	0.25
[d]-*B*	-	-	-	-	4	0.125	0.03125	0.03125
Recovered -*B* colonies								
*B*2-23-4	48h	ENR at 0.0625	Thr-86-Ala	Replacement	**32 to 128**	0.5	0.125	0.125
*B*2-5-2	48h	NOR at 8	Thr-86-Ile	Replacement	**128**	**16 to 32**	**4**	2
[d]-*R*	-	-	-	-	8	0.25	0.125	0.0625
Recovered -*R* colonies								
*R*1-21-2	96h	NOR at 2	Asp-90-Gly	Replacement	**256**	2 to 4	1 to 2	1
*R*1-4-1	48h	NAL at 128	Thr-86-Ile	Replacement	**512**	**64**	**16**	**8**
*R*1-11-1	48h	NAL at 32	Thr-86-Ala	Replacement	**128**	2	0.5	0.25 to 0.5
*R*1-16-1	96h	NAL at 32	-	-	**32 to 128**	1	0.25 to 0.5	0.25 to 1
*R*1-3-3	48h	CIP at 8	Thr-86-Ile	Replacement	**256 to 512**	**64**	**16**	**8**
[e]***C***	48h	CIP at 4	Thr-86-Ile	Replacement	**256**	**64**	**16**	**4**
Recovered *C* colonies								
*C*2-4-3	48h	CIP at 32	Thr-86-Ile, Asp-90-Asn	Replacement	**256**	**128**	**64**	**128**
*C*2-5-3	96h	CIP at 32	Thr-86-Ile	Replacement	**256**	**128**	**32 to 64**	**32 to 64**
*C*2-2-3	48h	CIP at 32	Thr-86-Ile	Replacement	**256**	**64 to 128**	**64**	**64 to 128**

P=parent strain, -*B*=*cmeB* insertionally deleted variant, -*R*=*cmeR* insertionally deleted variant, *C*=fluoroquinolone resistant variant
[a]Antimicrobial and the concentration of exposure.
[b]Effect of the sequenced single nucleotide substitutions for each isolate
[c]Numbers in bold represent isolates that were considered resistant. NARMS breakpoints (NAL 32µg/ml; NOR 16µg/ml; CIP 4µg/ml;) were used to classify isolates as resistant. ENR breakpoint were extrapolated from CIP NARMS (ENR 4µg/ml).
[d]Isolates were sequenced before exposure.
[e]Isolate was sequenced before second exposure.

Table 3.12 Mutant isolates that had their *gyrA* sequenced, antimicrobial profiles and sequence prediction

Sequences	Expected %	MIC(µg/ml)			
		NAL	NOR	CIP	ENR
Thr-86-Ile	73%(439)	128 to 512	16 to 128	4 to 64	2 to 128
Thr-86-Ala	15%(87)	32 to 128	0.5 to 2	0.125 to 0.5	0.125 to 0.5
Asp-90-Gly	6%(37)	256	2 to 4	1 to 2	0.5 to 1
[a]None (Nal R)	15%(87)	32 to 128	0.125 to 12.6	0.125 to 2	0.125 to 1
[b]None (Nal S)	5%(28)	4 to 24	0.125 to 12.6	0.125 to 2	0.0625 to 2.125
No Pattern	2%(13)	*Hard to Determine			

* Since the results were not conformed to any particular pattern it was hard to list them without confusion.

[a]No subistitutions were found at *gyrA* but the colonies were NAL resistant

[b]No mutations were found at *gyrA* but the isolate was overall susceptible.

CHAPTER 4

CONCLUSION AND FINAL DISCUSSION

Fluoroquinolone resistance in *Campylobacter* is of increasing concern in human medicine. The *Campylobacter* are able to circumvent the antimicrobial effect of fluoroquinolones by two basic mechanisms. First, an efflux pump exists within *Campylobacter* that allows the bacteria to expel various antibiotics, including the fluoroquinolones, from the bacterial cell before it can act. When this efflux pump is activated, even at a low level, a change in the regulator protein binding site can cause it to be over-expressed, creating elevated expulsion of antibiotics from the cell which renders the bacteria resistant to the antimicrobial. It is thought that a deletion of the regulator may not create resistance strains but it decreases susceptibility to various drugs especially when their resistance mechanisms are present. The second mechanism involves mutations in the *Campylobacter* gyrase (*gyrA*), a type II topoisomerase that is an important part of DNA replication due to its ability to open the replication fork. Alteration through point mutations at the QRDR of *gyrA* disrupts this process and is commonly observed in the presence of fluoroquinolones. Consequently, entry of the antimicrobial into the cell is inhibited, rendering bacteria with these mutations resistant.

In this body of research, the influence of both mechanisms was investigated which focused on the development of resistance through genetic mutations after exposure to fluoroquinolones. We used the Mutant Prevention Concentration (MPC) methodology, a technique that defines the concentration of an antibiotic which restricts selection of resistant mutants of a 10^{10} bacterial suspension. Four strains of *Campylobacter* were investigated, a parent strain (fully susceptible to fluoroquinolones), a strain with an

alteration in the efflux pump allowing over-expression of the pump, a strain with a mutation in the efflux pump rendering it inactive, and a strain with a mutation in *gyrA*, making it resistant to fluoroquinolone attachment at the subunit. Unlike previous investigations, we recorded MPC results at not only 48 hours of incubation but also at 96 hours. There were significant differences in the number of mutants observed at 48h and 96h in that more mutants were observed at 96 hours than at 48hours. The clinical relevance of this may also be significant as therapeutic administration is longer than 48 hours. Therefore, the reading of the MPC at 48h, which is considered standard, may not be sufficient to prevent resistant mutants from developing. Further, even after the MPC concentration was recorded at 96 hours, additional mutants were observed. These resistant 'survivors' represent a population of bacteria that can resist equal or higher levels of the drug the original strain was exposed to. This suggests that for some bacterial/drug combinations an MPC may not be so easily achievable. Consequently there may be serious clinical implications of evaluating MPC at only 48h, because that could still present a risk of resistance acquisition, although smaller than that seen with MIC based treatments.

The work described here raises additional questions that might be investigated in the future. First, genetic sequencing of the mutants could provide more insight into the most common genetic changes that occur following exposure to fluoroquinolones at various concentrations and the emergence of resistant strains. It is possible that one or more specific mutations occur more or less frequently depending upon the concentration of the antimicrobial that the bacteria is exposed to. This is important in the development

of mitigations to the development of antimicrobial resistance. By knowing the mutations that may happen, it can be possible to devise means to prevent their occurrence.

Sequencing of the *cmeRABC* operon to look for a *CmeR* biding site mutation is another area for future exploration. It is known that increased expression of the efflux pump may play a role in multiple antimicrobial resistances. In our study, exposing strains with a *cmeR* mutation to high concentrations of fluoroquinolones did not result in enhanced multiple antimicrobial resistance. This suggests that simple inactivation of the regulator genetic sequence may not be as effective as a mutation on its binding site at the *cmeR*-*cmeA* intergenic region, as has been shown by other researchers. However since we did not check for over-expression, but only checked for successful insertional deletion, there may not have been an increase of the efflux pump activity and another mechanism may have been responsible for the observed increase in resistance.

Many questions are still unanswered when it comes to use of the MPC methodology. As a result, it may take quite sometime before MPC translates into a treatment protocol which is implemented in clinical practice. Until then, MPC serves as a useful screening methodology for antimicrobials and study of their pharmacokinetics, providing insight into the various mechanisms a bacterium uses to evade killing or inhibition by antimicrobials.