Inhibition of the CmeABC efflux pump by antisense peptide nucleic acids reduces the emergence of spontaneous fluoroquinolone resistant mutants in *Campylobacter jejuni*

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

First and foremost, I would like to dedicate this dissertation to my mother Radhika Singh for her immense strength and unconditional support.

All the working women in my life have taught me the importance of perseverance and have encouraged me to be independent and follow my dreams.

For that reason, this dissertation is also dedicated to my grandmother C. Adilakshmi and my aunts, Gowri Nag and Jyotsna Chawla.

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ABSTRACT

Campylobacter is a major cause of bacterial gastroenteritis in humans and is commonly transmitted via undercooked poultry meat, unpasteurized milk and contaminated water. Over the years, *Campylobacter* has developed resistance to clinically important antibiotics including fluoroquinolones. Because of its significance in public health, both the Centers for Disease Control and Prevention and the World Health Organization has listed antibiotic resistant *Campylobacter* as a serious threat. Fluoroquinolone resistant mutants in *Campylobacter* occurs spontaneously and the resistance is mediated by the synergistic effect of the CmeABC multi-drug efflux pump and point mutations in the *gyrA* gene. Thus, antisense inhibition of *cmeABC* expression is a promising approach to combat fluoroquinolone resistance in *Campylobacter*. Previous studies have shown the specific inhibition of *cmeABC* expression by an antisense peptide nucleic acid (PNA) targeting the translational start of CmeA, but whether the PNA can be used as an adjuvant to potentiate fluoroquinolone antibiotics remains to be determined. Toward this end, *in vitro* and *in vivo* experiments were conducted to examine the efficacy of the PNA in reducing the emergence of spontaneous fluoroquinolone resistant mutants during treatment with a fluoroquinolone antibiotic. When fluoroquinolone-susceptible *Campylobacter* was treated with ciprofloxacin in culture media, resistant mutants emerged quickly and eventually replaced the susceptible population. However, addition of the PNA to the ciprofloxacin treatment prevented the emergence of resistant mutants completely, indicating the potentiating effect of the PNA on ciprofloxacin. Based on the *in vitro* results, the efficacy of the PNA was further evaluated *in vivo* using a *Campylobacter* infected chicken model. When given by oral gavage, the PNA was able to reduce, but unable to completely eliminate the emergence

of fluoroquinolone resistant mutants in *Campylobacter* in the intestinal tract of chickens treated with enrofloxacin. These results demonstrate the potentiating effect of the PNA on fluoroquinolone antibiotics and warrant additional studies to further optimize the anti-CmeABC approach as an adjunct therapy for antibiotic treatment of campylobacteriosis.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Campylobacter is a leading cause of gastroenteritis in humans. It is commonly present in the food production environments and is transmitted to humans via unpasteurized milk, contaminated water, and undercooked poultry meat. *Campylobacter jejuni* and *Campylobacter coli* are the most prevalent species causing campylobacteriosis. Clinical symptoms usually constitute acute watery or bloody diarrhea, nausea, fever, headache, and severe abdominal cramps. Although the infection is usually enteric and mild, there are cases of *Campylobacter*-induced severe complications, such as Guillain-Barré syndrome (GBS), Miller Fisher syndrome (MFS), reactive arthritis, bacteremia, meningitis, Urinary Tract Infections (UTIs) and cardiovascular complications.

Fortuitously, most *Campylobacter* infections are generally self-limiting and usually resolves itself within a few days. However, in cases where the patient is immunocompromised, elderly, young or pregnant, antibiotic treatment may be necessary. Some clinically severe cases with prolonged illness may also require antibiotic therapy, in these cases, fluoroquinolones and macrolides are the drugs of choice for therapeutic treatment. Unfortunately reports of antibiotic resistance in *Campylobacter* have been increasing drastically over the past decade. Fluoroquinolone resistance for instance has been on the rise all around the world. Prior to 1992, fluoroquinolone resistance in *Campylobacter* was very rare, however, recent reports from USA and other countries show a remarkable prevalence of fluoroquinolone resistant *Campylobacter*. *Campylobacter* has also been known to easily and rapidly develop resistance to fluoroquinolone antibiotics over the course of the treatment in patients with gastroenteritis. Because of the importance of *Campylobacter* as a zoonotic pathogen and its rising resistance to clinically

important antibiotics, both WHO and CDC have listed antibiotic-resistant *Campylobacter* as a serious threat to public health.

As a zoonotic pathogen, *Campylobacter* is exposed to antibiotics used for animal production and human medicine. When exposed to fluoroquinolone antibiotics, *Campylobacter* readily develops spontaneous resistant mutants that survive the treatment and eventually replace the fluoroquinolone susceptible *Campylobacter* under selection pressure. Fluoroquinolone antibiotics inhibit DNA gyrase in *Campylobacter* and resistance to this class of antibiotics is due to spontaneous point mutations in the *gyrA* gene. However, *gyrA* mutations alone are not sufficient and the function of the CmeABC efflux pump is also required for conferring clinically relevant resistance. The synergistic effect of the CmeABC efflux pump and point mutations in the *gyrA* gene lead to high levels of fluoroquinolone resistance. As the major efflux system in *Campylobacter*, CmeABC extrudes structurally diverse antibiotics including fluoroquinolones. Thus, inhibition of CmeABC represents a promising strategy for combating antibiotic-resistant *Campylobacter*.

To address the challenge with fluoroquinolone resistance in *Campylobacter*, various efforts have been made to target the CmeABC efflux pump. One of the strategies is to inhibit the expression of this efflux system by use of antisense peptide nucleic acid (PNA). PNA is a synthetic polymer that mimics nucleic acids, the ability to base pair with DNA and RNA allows PNA to perform antisense inhibition in a highly specific manner. Indeed, previous work has demonstrated that a PNA (CmeA1) targeting the translational start region of *cmeA* specifically inhibited expression of the *cmeABC* operon in *Campylobacter*. However, whether the CmeA1 PNA is able to reduce the emergence of fluoroquinolone resistant mutants in *Campylobacter* has not been examined. To close this knowledge gap and facilitate the development of an adjunct

therapy for fluoroquinolone antibiotics, we conducted both *in vitro* and *in vivo* experiments to evaluate the efficacy of the PNA in potentiating fluoroquinolone antibiotics.

In the first set of experiments, the efficacy of the CmeA1 PNA was examined in culture media. Treatment of *C. jejuni* with ciprofloxacin and the CmeA1 PNA completely prevented the emergence of fluoroquinolone resistant mutants, whereas treatment with ciprofloxacin alone generated resistant mutants which continued to multiply during the treatment and replaced the susceptible population at the end. The CmeA1 PNA alone did not affect the development of fluoroquinolone resistance. A scrambled sequence PNA (control) was also tested, the co-treatment with control PNA was unable to prevent the emergence of fluoroquinolone resistant mutants. These findings demonstrate that CmeA1 PNA is able to potentiate the killing effect of ciprofloxacin and prevented the emergence of fluoroquinolone resistant mutants in culture media.

In the second set of experiments using *Campylobacter*-infected chicken as a model system, we demonstrated that the CmeA1 PNA was able to initially reduce the emergence of fluoroquinolone resistant mutants in birds treated with enrofloxacin. However, the PNA was not able to completely eliminate the emergence of resistant mutants in the chicken intestinal tract. The results indicate the potential use of the CmeA1 PNA as an adjunct therapy for fluoroquinolone antibiotics, but additional studies are needed to optimize the treatment schemes.

Dissertation Organization

This dissertation is organized into five chapters, including a general introduction, a literature review, 2 chapters on experimental data, and a final summary. Chapter 1 is a general introduction for the Ph.D. project. Chapter 2 is a literature review of antibiotic resistance in *Campylobacter*, the function of the CmeABC efflux pump, and the potential of PNA in

inhibiting expression of CmeABC. Chapter 3 encompasses the *in vitro* experiments conducted to evaluate the efficacy of the PNA in culture media. Chapter 4 includes experiments assessing the *in vivo* efficacy of PNA using chickens as an animal model. Chapter 5 is the summary of this project which includes the general conclusions and future directions.

CHAPTER 2. LITERATURE REVIEW

Campylobacter

Campylobacter is a gram negative, curved rod bacterium. Most of the species are motile and have a single polar unsheathed flagellum at one or both ends. They are usually between 0.5 to 5 microns in length and 0.2 to 0.9 microns in width. They are microaerophilic and grow best in an atmosphere containing 5% - 10% oxygen (1). *Campylobacter* is best grown at 42 °C as they are thermophilic and do not multiply at temperatures below 30°C (2, 3). Some species, such as *Campylobacter jejuni,* can hydrolyze hippurate, while others, such as *Campylobacter coli,* are unable to. This phenotypic difference was often used to differentiate between *C. jejuni and C. coli* (4). However, currently PCR or MALDI-TOF MS techniques are routinely used to identify *Campylobacter* species (5, 6). More recently, whole genome sequence analysis of *Campylobacter* isolates has become routine, which provides high-resolution genomic data for molecular typing and phylogenic analysis of isolates from different sources (7, 8).

As an enteric organism, *Campylobacter* causes gastroenteritis in humans, and *C. jejuni* and *C. coli* are the two species commonly associated with the disease. The infectious dose of *C. jejuni* for humans is estimated to be between 500–800 organisms (9). In 1981 a British medical doctor, Robinson, was able to show this by swallowing 500 organisms in pasteurized milk. His results satisfied Koch's Postulates, proving the low infectious dose of *C. jejuni* (10).

The incubation period after ingestion of *C. jejuni* is 24-72 hours; however, in some cases it has been seen to last a week or longer (11). The most common clinical manifestation of infection is diarrhea, which can be quite severe. This is often accompanied by fever, abdominal pain and nausea. These symptoms are clinically indistinguishable from those caused by other enteric pathogens like *Salmonella* and *Shigella* and therefore diagnosis is made by isolating the

pathogen from stool samples (12). The disease is usually self-limiting and symptoms are resolved in a week; however, sometimes patients, particularly immunocompromised patients, have relapsing illness that lasts several weeks (13). Complications arising from *C. jejuni* infections can be quite severe, and heavy gastrointestinal hemorrhaging can occur. Pancreatitis and cholecystitis have also been reported in patients with severe complications from *C. jejuni* infection (14). Extraintestinal complications can also occur. For example, *C..jejuni* infection has been associated with Guillain–Barré syndrome (GBS) (1, 15-19), Miller Fisher syndrome (20), reactive arthritis (21, 22), bacteremia, septicemia, meningitis, Bell's palsy, Urinary Tract Infections (UTIs), and cardiovascular complications (15, 23, 24).

In cases when due to severity or because the patient is immunocompromised clinical treatment is needed, fluoroquinolones and macrolides are the first choices for antibiotic treatment of campylobacteriosis (25). However, Campylobacter is becoming increasingly resistant to clinically important antibiotics (26-34), compromising their effectiveness in clinical therapy. *Campylobacter* has been known to easily develop resistance to fluoroquinolone antibiotics over the course of the treatment, sometimes as rapidly as after 1 day of treatment in patients (35-37), rendering the treatment ineffective.

Fluoroquinolone resistant *Campylobacter* was very rare in the United States before 1992, however. this has changed over the years with reports of increasing prevalence of fluoroquinolone resistance amongst *Campylobacter* isolated from USA and Canada (38-40). Prevalence of fluoroquinolone resistant *Campylobacte*r have been increasing in European countries as well (41-46). In some reports of Asia and Africa, fluoroquinolone resistance rates have reached as high as 100% (33, 47, 48). Although there is a trend of increased macrolide

resistance in *Campylobacter*, the prevalence rates are generally lower than fluoroquinolone resistance (49-51).

With *C. jejuni*, fluoroquinolone-resistant mutants develop rapidly during treatment and the resistant population continues to persist even after removal of the antibiotic selection pressure, which suggests that no fitness cost is associated with the mutation leading to resistance (51-53). In contrast, macrolide-resistant mutants develop slowly, and the process involves a multistep development and requires prolonged exposure to the antibiotic (50). It has been observed that once the selection pressure is removed, the majority of the population reverts to macrolide-susceptible *Campylobacter* as macrolide-resistant mutants cannot compete with macrolide-susceptible *Campylobacter*, suggesting that there is a fitness cost associated with the resistance-conferring mutation (50, 51). The difference between fluroquinolone and macrolide in resistance development and fitness impact may explain why fluoroquinolone-resistant *Campylobacter* is much higher than macrolide-resistant *Campylobacter* worldwide.

Epidemiology, Etiology and Complications of Campylobacteriosis

Campylobacter is the leading cause of bacterial foodborne diarrheal disease worldwide. Symptoms can range from mild to serious including acute watery or bloody diarrhea, nausea, fever, headache, and abdominal pain with severe cramps (12, 24). *Campylobacter* has been identified as a common precursor to Guillain-Barré syndrome (GBS), an acute neuropathy (17, 18). The complication occurs post infection, when antibodies produced against the pathogen's surface structures cross-react with antigens on nerve endings. This autoimmune pathology leads to neuron damage that causes acute flaccid paralysis (54). GBS is the most common form of acute flaccid paralysis in the post-polio era (55). Miller Fisher syndrome (MFS), a variant of GBS, is also seen to be commonly associated with *Campylobacter* infections (56). Theses

neuropathies are a result of mimicry of the lipopolysaccharides (LPS) of *C. jejuni* to the gangliosides in peripheral neurons, leading to host antibodies attacking and damaging its own nerve cells (57). Acute reactive arthritis may also develop post *Campylobacter* infections (22). It is a spondyloarthropathy that usually develops within 4 weeks of the infection and is characterized by acute joint inflammation (58). The symptoms are generally joint and musculoskeletal related, but cardiac complications have also been observed (59, 60).

Campylobacteriosis is more common in children, the elderly and immunocompromised individuals (61). According to the Centers for Disease Control, there are about 1.3 million cases of *Campylobacter* infection each year in the United States, incurring medical cost between $1.3 to 6.8 billion dollars yearly (23, 62, 63). The incident reports of campylobacteriosis have also increased in Europe. Based on the Community Zoonoses Reports of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), *Campylobacter* has been the most commonly reported zoonotic pathogen in the EU, surpassing *Salmonella* and *Yersinia* (64, 65).

Although epidemiological data from Africa, Asia, and the Middle East are still limited and incomplete, a few studies conducted indicate that *Campylobacter* infection is endemic in these regions (33, 47). Due to population-level immunity in endemic regions, infection in developing countries is usually limited to children. It has been suggested that exposure in early life leads to the development of protective immunity (66-68). *C. jejuni* and *C. coli* are the most common species associated with *Campylobacter* enteritis in humans (69). A study conducted in hospitals of Yangzhou, China showed that 4.84% of 3,061 patients with diarrhea were PCR positive for *C. jejuni*, with the highest prevalence being detected in children younger than 7 years old (70). In another study conducted in Beijing, China, 14.9% (142/950) of patients with

gastroenteritis were reported to be positive for *Campylobacter* species (127 with *C. jejuni* and 15 with *C. coli*) (71). A report from Kolkata, India showed 7.0% (222/3186) of hospitalized patients with gastroenteritis were positive for *Campylobacter*, out of which 70% of the isolates were identified as *C. jejuni* (72). With the estimate that *Campylobacter* causes 400 to 500 million cases of diarrhea each year globally, it is undeniable that *Campylobacter* has a significant impact on global health (9). In fact, it was included as a pathogen of high concern in a report published by the World Health Organization in 2017 (73).

The intestinal tracts of food production animals are a reservoir for *Campylobacter* and the main source of infection for human campylobacteriosis is consumption of improperly prepared and contaminated animal foods such as poultry and raw milk (74-79). In the Netherlands cecal contents collected directly from chicken farms showed that 97% of the samples from layer farms and 93% samples from broiler farms were positive for *Campylobacter* (80). Screening of chicken meat from retail outlets in France displayed a high prevalence of *Campylobacter* (76% products positive) with the majority being *C. jejuni*. In Australia, *Campylobacter spp.* were detected in retail chicken meat (90%), lamb (38%), pork (31%) (81). Similarly in the US *Campylobacter* was detected in 76% of organic chicken products and 74% in conventional chicken products (82). Clearly, *Campylobacter* is highly prevalent in food production animals and are exposed to antibiotics that are routinely used on animal farms for growth promotion, disease prevention and control.

Studies show that conventional poultry farms have a higher percentage of antimicrobial resistant *Campylobacter* than organic farms where antimicrobials are not used (83). *Campylobacter* is a zoonotic pathogen and is therefore exposed to antibiotics used in both animal production and human medicine. This has raised concerns about using antibiotics important in

human medicine on animal farms as it may select antibiotic resistant *Campylobacter* that is transmitted to humans via the food chain. In 2005, the Food and Drug Administration banned the use of fluoroquinolones on poultry farms in the United States (32, 84). European countries have also implemented bans on usage of subtherapeutic doses of antibiotics as a feed additive to promote growth in food production animals.

Despite these measures, antimicrobial resistance in *Campylobacter* continues to persist and antibiotic-resistant *Campylobacter* is still on the rise (51, 85, 86). Patients infected with fluoroquinolone resistant *Campylobacter* tend to have a prolonged duration of diarrhea when compared to patients infected with fluoroquinolone susceptible *Campylobacter* (87). Fluoroquinolone resistance has been increasing rapidly in the US and Canada (29), even though it was rare before 1992 (51). Similarly, European countries have also seen the rise of fluoroquinolone resistant *Campylobacter*, with Spain reporting 72% *Campylobacter* was fluoroquinolone resistant (88), Greece reporting 55% (45), Finland reporting 46% (44), and Germany reporting 30% (42). In Thailand, over 80% *Campylobacter* isolates are resistant to fluoroquinolones (51, 88). Unlike the drastically elevated prevalence of fluoroquinolone resistance, macrolide resistance in *Campylobacter* is still relatively low (10% or lower in the US); however, it is on the rise in Asia and Africa (51, 89). In Korea, macrolide resistance was reported to be present in 14 - 50% of the *Campylobacter* isolates (90, 91), and 17% was reported from Thailand (92), Tetracycline resistance amongst *Campylobacter* isolates are high all around the world (93-95).

Antibiotic Resistance Mechanisms in *Campylobacter*

Campylobacter displays intrinsic resistance to novobiocin, bacitracin, vancomycin, β-lactams, and polymyxins due to the absence of antibiotic targets and/or the low affinity of these

antibiotic to target sites (96). Thus, these antibiotics are rarely used for clinical treatment of campylobacteriosis. Although fluoroquinolones, macrolides, tetracyclines, and aminoglycosides are effective against *Campylobacter*, acquired resistance to these antibiotics is increasingly reported. *Campylobacter* can confer resistance to these antibiotics through multiple mechanisms.

Blocking antibiotics from reaching their target is a simple mechanism used by *Campylobacter* for resistance. By altering membrane permeability *Campylobacter* can restrict the entry of antibiotics into the cells. For example, modulating the expression of *porA*, encoding the major outer membrane porin (MOMP), can affect the permeability of the membrane, which prevents the uptake of certain antibiotics such as macrolides and most β-lactams (96). Another example of restricting entry of antibiotics into the cell is through the production of lipooligosaccharide that confers resistance to hydrophobic drugs. The outer membrane and surface polysaccharides are essential components that determine the permeability of the cell, and knocking out the lipooligosaccharide (LOS) made *Campylobacter* more susceptible to antibiotics (97).

Enzymatic deactivation of antibiotics is also a mechanism employed by *Campylobacter* for antibiotic resistance. Chloramphenicol inhibits bacterial protein synthesis by preventing the elongation step when it binds to the 50S rRNA. This can be countered by the *cat* gene that encodes an acetyltransferase, which modifies chloramphenicol to prevent it from binding to ribosomes (98). This plasmid borne chloramphenicol resistance gene has been reported in *Campylobacter* (99). β-lactam antibiotics are also susceptible to enzymatic degradation from β-lactamases. These enzymes are encoded by a variety of different genes that are easily acquired by *Campylobacter*. Some common genes identified in *Campylobacter* include OXA-type β-lactamases and metallo-β-lactamases (100).

Target protection is a well-established mechanism for antibiotic resistance present in many pathogenic bacteria. Tetracyclines bind to ribosomes and interfere with the elongation step during peptide synthesis by inhibiting aminoacyl-tRNA binding to the mRNA-ribosome complex (101). Tetracycline resistance in *Campylobacter* is conferred by the *tetO* gene that encodes the Tet(O) protein, which protects against tetracycline binding to ribosomal A site. Tet(O) is a member of a class of proteins called ribosomal protection proteins or RPPs. Other members of this class of proteins include Tet(M), Tet(S), Tet(T) and Tet(Q) (102). Evidence shows that Tet(O) is acquired by *Campylobacter* through horizontal transfer (103).

Target modification is another important mechanism in *Campylobacter* and is responsible for resistance to 2 classes of important antibiotics. *Campylobacter* easily acquires mutations in the 23S rRNA leading to erythromycin resistance. The A2075G mutation in the 23S rRNA has been identified as a prevalent mutation contributing to high-level erythromycin resistance in *Campylobacter* (104, 105). Point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene is responsible for ciprofloxacin resistance in *C. jejuni* and *C. coli* (106). DNA gyrase is essential for bacterial growth (107). Fluoroquinolone antibiotics such as ciprofloxacin bind to DNA gyrase while it is attached to the DNA to form a stable complex that traps the enzyme in place, this complex inhibits progression of bacterial DNA synthesis leading to bacterial death(108). DNA gyrase has two subunits encoded by the genes *gyrA* and *gyrB* and certain point mutations in *gyrA* lead to *Campylobacter* resistance to fluoroquinolones. Additionally, these mutations do not seem to alter the function of DNA gyrase, which means there isn't a fitness cost associated with the acquisition of the resistance (51, 53, 109). This phenomenon could explain why fluoroquinolone resistance has persisted on poultry farms despite the withdrawal of the antibiotics in 2005. As mentioned below many studies showed that

fluoroquinolone resistant *Campylobacter* is still prevalent on poultry farms in the united states even after the fluoroquinolone ban (85). Since the acquisition of the resistance does not reduce the fitness of *Campylobacter*, fluoroquinolone-resistant *Campylobacte*r is expected to continue to persist even in the absence of antibiotic selection pressure (28, 109, 110).

Another major mechanism for antimicrobial resistance in *Campylobacter* is reduced intracellular accumulation of antibiotics via efflux, such as the multidrug efflux pump CmeABC that confers resistance to fluoroquinolones, macrolides, tetracyclines, florfenicols, and β-lactams as well as other toxic compounds (28, 111). Since it gives rise of a multidrug resistance phenotype, this mechanism is extremely concerning as it severely restricts the options for anti-microbial therapy. Furthermore, CmeABC work in synergy with the other mechanisms mentioned above to further elevate the resistance levels (34, 87, 96, 105, 112). The synergistic effect between efflux and the acquisition of resistance genes and mutations further compromise the effectiveness of therapeutic strategies. For these reasons, the CmeABC efflux pump and its potential inhibition are discussed in more details below.

Multidrug Efflux Pumps in *Campylobacter*

Multidrug efflux pumps enable *Campylobacter* to actively resist antibiotics and constitute a vital mechanism of antibiotic resistance (111-114). Multidrug transporters can be differentiated based on their energy sources. ATP-binding cassette (ABC) transporters use ATP as an energy source, while secondary transporters use a transmembrane electrochemical gradient of protons or sodium ions to facilitate transport (115). Secondary multidrug transporters are further categorized into four superfamilies based on their structural properties. These include the resistance–nodulation–division (RND) family consisting of three proteins located in three different cellular compartments (inner membrane, periplasm and outer membrane), the major

facilitator superfamily (MFS) that consists of 12–14 transmembrane domains, the multidrug and toxic compound extrusion (MATE) family which also has 12 transmembrane domains but with significant sequence differences from MFS transporters, and the small multidrug resistance (SMR) family comprising of small proteins usually containing 4 TMs (115, 116).

Two multidrug efflux pumps, CmeABC and CmeDEF, have been characterized in *Campylobacter*, both belonging to the RND family of transporters. CmeABC is the major efflux pump in *C. jejuni* and contributes to resistance to a variety of antimicrobials. Unlike CmeABC, CmeDEF has only a moderate effect on antimicrobial resistance. CmeDEF interacts with CmeABC in maintaining cell viability (113). Sequencing of the genomic DNA of *C. jejuni* NCTC 11168 revealed 14 putative drug efflux transporters of different families, but most of these transporters remain unknown in terms of function (116). Most of these transporters, however, do not show a synergistic effect with other resistance mechanisms like CmeABC does (114).

The CmeABC multidrug efflux pump is an energy dependent RND type efflux pump. It has three subunits CmeA, CmeB and CmeC. The CmeA subunit is a periplasmic fusion protein, CmeB is the efflux transporter present in the inner membrane, and CmeC is the outer membrane protein (111, 117). These three subunits form a channel in the membrane of the bacteria to extrude antimicrobials and toxic compounds or metabolites. Therefore, CmeABC is not just responsible for broad spectrum resistance to antimicrobial agents in *Campylobacter*, it is also necessary for *Campylobacter* colonization in the intestinal tract of animals as it also expels bile salts (118, 119). The efflux pump is encoded by a three gene operon, *cmeA*, *cmeB* and *cmeC*, with only one promoter in front of *cmeA*. The peptide sequence of CmeB shows 41% similarity to AcrB (111), which is a major efflux pump in *Escherichia coli* (120). Furthermore, *cmeABC* is

subject to regulation by CmeR, a 210-amino-acid protein encoded by *cmeR* upstream of *cmeA*. Studies showed that CmeR is a transcriptional regulator of the TetR family, binds to the promoter of *cmeABC*, and represses expression of this efflux system (121, 122).

Bile salts also have a significant impact on the expression levels of *cmeABC*. Evidence shows that transcription of *cmeABC* is upregulated in the presence of Bile salts, so is the production of the CmeABC proteins (119). This increase is due to the fact that bile salts inhibit the binding of CmeR to the promoter of *cmeABC*, liberating the operon from repression (119). Increase in expression levels of the CmeABC Efflux pump in the presence of bile salts modestly increases antimicrobial resistance levels in *Campylobacter* (119, 123, 124).

Another transcriptional regulator for the CmeABC Efflux pump is CosR. CosR is an essential response regulator in *C. jejuni* and regulates many genes involved in cellular functions such as lipid metabolism, protein synthesis, and energy production, etc (125). Initially transcriptomic analysis showed an increase in transcription levels of *cmeA*, *cmeB* and *cmeC* when CosR was knocked down. Further experiments demonstrated that CosR directly binds to the *cmeABC* promoter, and the binding site is 17bp upstream to the CmeR binding site (125). This finding indicates that expression of *cmeABC* is dynamically regulated by multiple factors. As expression of this large efflux pump can be costly for *Campylobacter*, constitutive expression is only at a modest level, but enhanced expression of *cmeABC* occurs in the presence of toxic compounds (such as bile and antibiotics), facilitating the adaptation of *Campylobacter* to environmental changes (121-123).

CmeABC functions synergistically with other mechanisms in conferring clinically relevant antibiotic resistance. For example, the efflux pump is necessary for acquiring and maintaining fluoroquinolone resistance and for the emergence of fluoroquinolone resistant

mutants in *C. jejuni* because *gyrA* mutations alone are not sufficient for resistance to the antibiotics (114, 124, 126). Similarly, CmeABC efflux pump works in synergy with target mutations in the ribosomal proteins to confer resistance to macrolides in *Campylobacter* (127). The importance of CmeABC efflux pump in mediating antibiotic resistance has been clearly shown, providing a strong rationale for inhibition to reduce the emergence of antibiotic-resistant *Campylobacter*.

Inhibition of the CmeABC Efflux Pump

Owing to their key role in antibiotic resistance, inhibition of multidrug efflux transporters represents a promising strategy to combat antimicrobial resistance. Unsurprisingly, many studies have been performed to identify small molecule inhibitors to interfere with the activity of efflux pumps with the intention of improving the usefulness of antibacterial agents already in use (128-135). These Efflux Pump Inhibitors or EPIs are being extensively researched for broad-spectrum inhibition of efflux pumps, particularly in gram negative bacteria. While the strategy of inhibiting efflux pumps to re-sensitize the resistant bacteria to clinically available antibiotics is a good one, these EPIs tend to be toxic at the concentrations needed for inhibition and have therefore not been put into use (128). Furthermore, EPIs do not always produce consistent inhibition against different efflux pumps and are therefore not as broad-spectrum as initially believed (132-134, 136). Many studies were conducted to reduce or eliminate the toxic effects of EPIs, but were met with limited success: either the structural changes reduce the inhibition potency of the EPIs or it is unable to negate the toxic side effects (137-140).

Different from EPIs that block the function of efflux pumps, another strategy is to inhibit the expression of efflux transporters. A novel synthetic molecule called peptide nucleic acid or PNA has been successfully used to knock down expression of the CmeABC efflux pump and re-

sensitize antibiotic-resistant *Campylobacter* to clinically relevant antibiotics like fluoroquinolones and macrolides that are used to treat *Campylobacter* infections (141, 142).

Peptide Nucleic Acids

PNA was first created in the laboratory of organic chemist Prof. Ole Buchardt and biochemist Peter Nielsen during the 1980s (143). It is a synthetic polymer with a backbone composed of N-(2-aminoethyl) glycine units instead of a sugar-phosphate backbone naturally present in nucleic acids (144). It has the nucleobases attached to the glycine nitrogen via carbonyl methylene linkers and can base pair with DNA and RNA using the Watson Crick hydrogen bonding scheme (143-146). PNA oligomers bind with high sequence discrimination or specificity to complementary oligomers. Due to the N-(2-aminoethyl) glycine backbone, PNA molecules are neutral, unlike negatively charged DNA and RNA, therefore they are able to form stable duplexes and triplexes with DNA and RNA with greater affinity since they lack electrostatic repulsion.

PNA is also resistant to enzymatic degradation and therefore very stable within a cell (147). However, entry into cells is a challenge and they must be conjugated to a delivery mechanism. HPLC analysis of PNA in human serum and cellular extracts confirms stability of PNA in the presence of proteolytic enzymes (148). PNA injected intravenously in rats was detected in all major organs, such as kidney, liver, and spleen, and was recovered from the urine 24 hours after it was administered (149). This data demonstrates that PNA is extremely stable in diverse conditions.

PNA molecules have been used for various applications as a tool in molecular biology and biotechnology. Due to the high specificity, PNA can discriminate between single base pair mismatches, and this high sequence discrimination is an invaluable tool in molecular biology

(150-152). They have also been used for development of biosensors and for diagnostics purposes. For example, PNA was designed to target microRNAs (miRs) that are aberrantly expressed in prostate cancer. These microRNA levels can be detected in biofluids, making them an ideal noninvasive biomarker for diagnosis (153). Fluorescent in situ hybridization (FISH) can also be performed using PNA probes (154). PNA is able to distinguish point mutations with high specificity (155). This characteristic was exploited to detect clarithromycin resistance in *Helicobacter pylori* suspensions (156). It is a rapid way to detect resistance without having to use fastidious culturing methods.

PNA has great potential in helping us combat multidrug resistant *Campylobacter* as it can serve as a targeted antisense therapeutic (157, 158). As mentioned earlier however, PNA cannot easily gain entry into cells, and to facilitate their uptake, they must first be linked to cell penetrating peptides or CPPs. Many cell-penetrating peptides have been documented over the years. They are fairly short (< 30 amino acids) and can translocate various cargos into cells (159, 160). A PNA molecule targeting a specific gene for antisense inhibition conjugated to an appropriate CPP can be potentially used as an antisense therapeutic.

PNA has been successfully used to inhibit bacterial growth in *Escherichia coli* by targeting functional sites of rRNA. The effect is similar to antibiotics that inhibit translation like tetracycline. Control PNAs, i.e. PNAs with unrelated or mismatched sequences show no inhibitory effect (161). In an effort to develop PNA as a broad spectrum therapeutic, a study was conducted to inhibit growth in all gram-negative bacteria by targeting the *rpoD* gene. The anti-*rpoD* PNAs displayed bactericidal effect against multidrug-resistant *Escherichia coli, Salmonella enterica, Klebsiella pneumoniae,* and *Shigella flexneri in vitro* and *in vivo*. This study was also able to demonstrate that treatment with PNA of infected human gastric mucosal epithelial cells

exhibited the complete inhibition of bacterial growth and had no influence on morphology and growth of human mucosal cells (162).

PNAs have been used to inhibit a multi-drug resistant *K. pneumoniae* strain. In this case, PNA was designed against 2 essential genes *gyrA* and *ompA*. The PNAs alone did not permeate the bacteria, however, when conjugated to the $(KFF)_3K$ cell penetrating peptide, they were able to inhibit the growth of the MDR *K. pneumoniae* at concentrations of 20 μM and 40 μM, respectively. The bactericidal effects were seen within 6 hours. This experiment was also conducted in MDR *K. pneumoniae* infected human cell lines, the CPP-PNAs were able to cure the infection with no noticeable toxicity to the human cells (163). A very similar study was conducted in MDR *Acinetobacter baumannii*, where PNA was designed to target *carA*, an essential *Acinetobacter* gene. *In vitro* testing was conducted with four clinical strains of MDR *A. baumannii* and their PNA construct was able to inhibit bacterial growth at a concentration of 1.25 μM. *In vivo* testing was conducted in the *Galleria mellonella* model of sepsis. Two doses (5 μM and 20 μM) of the CPP-PNA targeting the essential gene (*carA*) were evaluated. The results demonstrated that the low dose of PNA did not have any effect but the high dose of the CPP-PNA was able to significantly reduce the mortality rate of the caterpillars (164). The use of PNA and other antisense oligonucleotides as bactericidal agents is being researched as an alternative for the antibiotics currently being used (165).

Broad spectrum PNAs, however, kill pathogenic and non-pathogenic bacteria indiscriminately and it is also likely that targeting an essential gene will lead to the development and selection of resistant mutants as it has happened with antibiotics. Targeting resistance mechanisms with PNA to revert the bacteria to be susceptible is thus becoming a popular strategy. Colleen M. Courtney and Anushree Chatterjee were able to re-sensitize drug-resistant

Escherichia coli to β-lactam antibiotics by using targeted antisense translational inhibition of the TEM-1 β-lactamase transcript with PNA (166).

In *Campylobacter*, PNA targeting the CmeABC efflux pump has been used successfully to knock down the expression of this multidrug efflux system. The optimal PNA was identified by selecting the best sequence for antisense inhibition of the translational initiation site of *cmeA* (141, 142, 167). The *cmeA*-specific PNA significantly reduced the expression level of CmeABC as seen by Western blotting analysis and decreased the minimum inhibitory concentration (MIC) of antibiotics. Although PNA has shown the potential to inhibit CmeABC and consequently sensitize *Campylobacter* to antibiotics, it requires further development before it can used as therapeutic adjuvant. Unlike EPIs, PNA does not seem to have significant toxic effect, as *in vivo* studies conducted in a mouse model demonstrated no toxic effect (143).

Conclusions and Future Directions

Campylobacter is a significant burden to public health with fewer treatment options as it has developed resistance to clinically important antibiotics. As a highly adaptable bacterial organism, *Campylobacter* possesses multiple mechanisms for antibiotic resistance. Of particular note is the multidrug efflux transporter CmeABC, which confers broad resistance to antibiotics and toxic compounds. By targeting the multidrug resistance system of *Campylobacter*, it is possible to potentiate the effectiveness of currently available antibiotics while minimizing the rapid emergence of fluoroquinolone resistant mutants often seen in patients receiving treatment for Campylobacteriosis. Toward this direction, PNA has shown a great potential to specifically inhibit the expression of the *cmeABC* efflux pump and work as an adjunct therapeutic. However, the previous work was done in culture media, and further studies are needed to determine the efficacy of PNA in inhibiting the expression of CmeABC under various conditions and the

emergence of antibiotic resistant mutants during antibiotic treatment. Additionally, its potency needs to be evaluated *in vivo* to determine whether it is effective in inhibiting CmeABC of *Campylobacter* in the intestinal tract, where the environment is much more complex that in culture media.

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CHAPTER 3. INHIBITION OF THE CmeABC MULTIDRUG EFFLUX PUMP BY PEPTIDE NUCLEIC ACID PREVENTS THE EMERGENCE OF SPONTANEOUS FLUOROQUINOLONE RESISTANT MUTANTS IN *CAMPYLOBACTER JEJUNI IN VITRO*

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Abstract

Campylobacter jejuni is a major enteric pathogen that has become resistant to clinically important antibiotics. The CmeABC efflux pump is one of the major resistance mechanisms that has been known to work in synergy with point mutations in the *gyrA* gene to confer high-level fluoroquinolone (FQ) resistance, which develops rapidly when *Campylobacter* is exposed to FQ antibiotics that are used for clinical therapy of campylobacteriosis. Previously it has been shown that peptide nucleic acid (PNA CmeA1) specifically targeting the predominant drug efflux transporter CmeABC inhibited the expression of this efflux pump and sensitized *Campylobacter* to clinically important antibiotics including FQs. In this study, the CmeA1 PNA was evaluated to inhibit the emergence of fluoroquinolone resistant mutants. A FQ-susceptible *Campylobacter jejuni* strain was subjected to treatments with ciprofloxacin, with or without the PNA. After 48 hrs, the culture treated with ciprofloxacin alone developed FQ-resistant mutants, while the culture treated with ciprofloxacin and the PNA showed no growth. To further quantify the difference, growth kinetics were measured at different time points. Once ciprofloxacin was added, the colony forming units (CFUs) of the culture without PNA decreased, but between 12 and 24 hrs, FQ-resistant mutants emerged and the CFUs started to increase. On the contrary, the CFUs of the culture treated with ciprofloxacin and PNA steadily decreased and were no longer

detectable after 12 hrs, indicating that no resistant mutants emerged in the culture. These results show that the anti-CmeABC PNA potentiates the killing effect of ciprofloxacin and inhibits the emergence of ciprofloxacin resistant mutants in *C. jejuni*.

Introduction

Campylobacter has been recognized as a leading cause of bacterial food-borne disease. According to the Centers for Disease Control (CDC), there are over 1.3 million cases of campylobacteriosis each year in the United States (1). Worldwide, *Campylobacter* infections are estimated to cause 400-500 million cases of diarrhea each year (2). A major source of infection is consumption of contaminated animal food like poultry and raw milk. Clinical symptoms can range from mild to severe, including bloody diarrhea, nausea, fever, headache and abdominal pain. Extra-gastrointestinal complications may also develop, which include Guillain-Barré syndrome (GBS) (3-5), Miller Fisher syndrome (6), bacteremia and septicemia as well as reactive arthritis (7, 8), meningitis, and cardiovascular complications (9). When clinical therapy is needed, fluoroquinolones and macrolides are the first line of antibiotics used to treat campylobacteriosis; however, resistance to these antibiotics, particularly to fluoroquinolones, has increased drastically over the past decades (10, 11).

Campylobacter possesses multiple mechanisms for antibiotic resistance. It is intrinsically resistant to novobiocin, bacitracin, vancomycin, and polymyxins due to lack of target sites or low affinity for them. *Campylobacter* is also able to alter membrane permeability to restrict the uptake of antibiotics like macrolides and β-lactams (12). Synthesizing enzymes to degrade or modify antibiotics is another common resistance mechanism and the resistance genes are normally obtained via horizontal gene transfer (13, 14). *Campylobacter* may also protect or modify the drug target by acquiring various resistance genes or through mutations in existing

genes (15-17). For example, point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene is responsible for fluroquinolone resistance in *C. jejuni* and *C. coli*. Fluoroquinolone antibiotics bind to DNA gyrase while it is attached to the DNA to form a stable complex, which traps the enzyme in place leading to double stranded breaks in the DNA and results in bacterial death. Certain point mutations in *gyrA* result in the inability of fluoroquinolones to bind to their target, leading to resistance. Additionally, these resistance-conferring mutations do not seem to alter the function of DNA gyrase, which means there isn't a fitness cost associated with the acquisition of the resistance (18-21), but it is usually the case with macrolide resistance (22).

One of the primary mechanisms of antimicrobial resistance in *Campylobacter* is antibiotic efflux pumps such as CmeABC (23). The CmeABC efflux pump is encoded by a three-gene operon (*cmeA*, *cmeB*, and *cmeC*) and is made of three proteins; an inner membrane transporter (CmeB), a periplasmic fusion protein (CmeA), and an outer membrane protein (CmeC). CmeABC confers resistance to structurally and functionally diverse antibiotics and toxic compounds (24). Furthermore, it works in synergy with the other resistance mechanisms mentioned above (17, 25). It has also been shown that overexpression of CmeABC in *Campylobacter* significantly increases the frequency of emergence of fluoroquinolone-resistant mutants under selection pressure (26). The synergistic effect of efflux pumps and the acquisition of resistance genes and mutations poses a significant challenge for clinical therapy. This is especially obvious with fluoroquinolone antibiotics as resistance to this class of antibiotics occurs during clinical treatment due to spontaneous mutations in *gyrA* and the synergistic function of CmeABC. Thus, inhibition of CmeABC is a promising strategy to reduce the emergence of fluoroquinolone resistant mutants as well as potentiate the efficacy of antibiotics.

One way to inhibit the function of CmeABC is to use antisense peptide nucleic acids (PNA) to inhibit the expression of this efflux pump. PNA is a synthetic polymer composed of N-(2-aminoethyl) glycine units with nucleobases attached to the glycine nitrogen via carbonyl methylene linkers. It can, therefore, base pair with DNA and RNA with high specificity (27-30). PNA is resistant to enzymatic degradation and remains in tissues for long periods of time (31, 32). These properties of high specificity of binding and stability in the presence of proteolytic enzymes make PNA uniquely suitable for use as an antisense therapeutic. Previously, it has been shown that PNA designed to target *cmeABC* can significantly reduce the expression of the efflux pump and consequently decrease MICs of antibiotics (33). Particularly, the PNA sequence spanning the ribosome binding site (RBS) of *cmeA* was the most successful at inhibiting expression of the cmeABC efflux pump (34).

Despite this progress, it remains to be determined whether the PNA against CmeABC can inhibit the emergence of fluoroquinolone resistant mutants in *Campylobacter* during antibiotic treatment. Since the PNA specifically inhibits the expression of *cmeABC*, we hypothesized that use of PNA along with a fluoroquinolone antibiotic would prevent the emergence of fluoroquinolone resistant mutants. To test this hypothesis, we conducted antibiotic treatment experiments in the presence or absence of PNA and compared the rates of emergence of fluoroquinolone resistant mutants using an *in vitro* culture system.

Materials and Methods

Bacterial Strains and Growth Conditions

C. jejuni strains were cultured microaerobically in a gas chamber (AnaeroPack, ThermoFisher) filled with 5% O_2, 10% CO_2 and 85% N_2 on Mueller-Hinton (MH) agar or in

Mueller-Hinton (MH) broth at 42°C. All strains are preserved as 30% glycerol stock at -80°C. The strains used in this study are listed in the table below.

Table 1. Bacterial strains used in this study and the minimum inhibitory concentrations of ciprofloxacin.

Bacterial Strain	**Genotype or Phenotype**	**MIC for Cipro**	**Reference**
NCTC 11168	*C. jejuni* reference strain	0.063 µg/ml	(35)
11168W199G	Naturally occurring variant of 11168 with point mutation in *mutY*	0.063 µg/ml	(36)
CT6:16L	*C. jejuni* isolate from commercial turkey	0.063 µg/ml	(37)
CT7:20C1	*C. jejuni* isolate from commercial turkey	0.063 µg/ml	(37)
11168W199GCRM1 - 11168W199GCRM10	Ciprofloxacin resistant mutants generated by applying antibiotic selection pressure on 11168W199G	4 µg/ml	This Study

Antimicrobial Susceptibility Tests

Ciprofloxacin, a fluoroquinolone antibiotic, used in this study was purchased from Sigma-Aldrich Co. LLC. Its MIC was determined using the standard broth microdilution method as previously described (23). Briefly, two-fold serial dilutions of ciprofloxacin were made in MH broth in wells of 96-well round bottomed culture plates. The first well contained only MH broth as a no-antibiotic control. Each antibiotic dilution was 50 µl in volume. The *C. jejuni* inoculum

was prepared by growing a fresh culture overnight on MH agar plates. The culture was then resuspended in MH broth to obtain OD 0.1 at 600 nm. Subsequently it was diluted 100-fold to achieve approximately 10^6 CFU/ml., and 50 µl of the cell suspension was then added to each of the wells containing various concentrations of antibiotics. The plates were incubated at 42°C for 24 hrs in a microaerobic chamber. Each MIC test was run in duplicates.

To evaluate the effect of PNA on potentiating the effect of antibiotics, the MIC assay was performed in parallel with or without the CmeA1 PNA. The *C. jejuni* inoculum was prepared as described above and split into two parts: one with PNA (2 µM for 11168 and 1 µM for CT strains) and the other without PNA. After 30 mins, the cell inocula were added to the 96-well plates containing various ciprofloxacin dilutions. The plates were incubated at 42°C for 24 hrs microaerobically.

PNA Preparation

The CmeA1 PNA was selected for this study based on the fact that it was the most effective in inhibition of *cmeABC* (34). The PNA targets the ribosome-binding site (RBS) of *cmeA*. The PNA was synthesized and conjugated to a penetrating peptide (CPP) by PNA BIO INC (Newbury Park, CA, USA), resulting in the CPP-PNA construct: KFFKFFKFFK-tgccttgaaaaa. A second control PNA, not targeting any specific sequences, in *Campylobacter* was also synthesized in the same way: KFFKFFKFFK-acacacacacac. Each PNAs was received as a lyophilized powder, which was reconstituted using sterilized dd.H_2O to make a stock solution of 200 µM and aliquoted into tubes for storage at -20°C.

Qualitative Determination of Emergence of Ciprofloxacin Resistant Mutants

A qualitative growth test was performed with antibiotic selection pressure to measure the emergence of ciprofloxacin resistant mutants in the presence or absence of the CmeA1 PNA. A 10^6 CFU/ml starting culture was prepared from *C. jejuni* 11168W199G. This strain was used in

place of *C. jejuni* NCTC 11168 as it has a point mutation in the *mutY* gene that increases its mutation frequency by 100-fold when compared to wild-type 11168 (36). This gives rise of a large dynamic range of resistant mutant emergence that can be measured easily in culture media. A fresh culture of 11168W199G was divided into 5 different treatment groups as listed below:

- Treated with ciprofloxacin (1 µg/ml)
- Treated with ciprofloxacin (1 µg/ml) and the CmeA1 PNA (2 µM)
- Treated with the CmeA1PNA (2 µM) only
- Treated with ciprofloxacin (1 µg/ml) and the control PNA (2 µM)
- Treated with the control PNA (2 µM) only

The appropriate amount of PNA was added to the cultures. After 30 min incubation, ciprofloxacin was added and 100 µl of the mixture was distributed to a well of a 96-well plate. The ciprofloxacin-only treatment had 16 wells, while every other treatment had 8 wells. The plates were incubated at 42 °C microaerobically in a gas chamber. At 24 and 48 hrs of incubation, visual observation of growth indicated by turbidity change was conducted and images of the growth were taken. To confirm growth or lack thereof, at the end of the experiment (after 48 hours of incubation), the cultures in the wells were spread onto MH agar plates with or without ciprofloxacin (1 µg/ml) and bacterial growth on plates was observed after 24 hrs of incubation.

Quantitative Determination of the Emergence of Ciprofloxacin Resistant Mutants

Growth kinetics of *C. jejuni* in the presence of ciprofloxacin with or without PNA were conducted in culture media to determine the frequencies of emergence of ciprofloxacin resistant mutants. The starting culture (10^6 CFU/ml) of *C. jejuni* 11168W199G was prepared and divided into 3 treatment groups, including ciprofloxacin-only treated group, ciprofloxacin and

CmeA1PNA treated group, and CmeA1PNA-only treated group. The cultures of various treatments were grown at 42 °C microaerobically on 96-well round bottomed culture plates. Each treatment group had 15 wells and at each of the time points (0, 6, 12, 24 and 48 hrs), cultures collected from 3 wells were used to measure the colony forming units (CFUs). The CFUs were determined using the drop plating method described below.

To further confirm the effect of the CmeA1 PNA in reducing the emergence of fluoroquinolone resistant mutants, an additional growth kinetics test was conducted using culture tubes instead of 96-well plates to allow continuous sampling of the same cultures at different time points. The starting culture (10^6 CFU/ml) was prepared and divided into 3 treatment groups: ciprofloxacin-only treated group, ciprofloxacin and CmeA1 PNA treated group, and CmeA1PNA-only treated group. The tubes were incubated at 42 °C microaerobically. At 0, 6, 12, 24, and 48 hrs, 100 µl of culture was collected from each tube to determine the CFU numbers. This experiment was performed in triplicates, i.e. 3 tubes were used for each treatment condition. The CFU counts were determined by using the drop plating method as described below.

Drop Plating Method for CFU Enumeration

C. jejuni cultures collected from different treatment groups and time points were diluted to make 10-fold dilution series. From each dilution, three 10 µl drops were plated on an MH agar plate, 7 dilutions along with the original were plated in this manner to obtain the best dilution for counting single colonies. The colonies were counted, and the CFU/ml of the original culture was calculated based on the dilution factors.

Statistical Analysis

An unpaired student's t test was used to compare the average *Campylobacter* CFU/ml between treatment groups. *P* values of < 0.05 were deemed to be statistically significant. GraphPad prism 8 software (GraphPad Software San Diego, CA) was used to generate the graphs.

Determination of *gyrA* Mutations

In *Campylobacter*, fluoroquinolone resistance is conferred by point mutations in the *gyrA* gene in concurrence with the CmeABC efflux pump (38). To confirm the mechanism of fluroquinolone resistance in this study and for determination of the point mutations in the quinolone resistance-determining region (QRDR) of *gyrA*, primers GyrAF1 (5′-CAACTGGTTCTAGCCTTTTG-3′) and GyrAR1 (5′-AATTTCACTCATAGCCTCACG-3′) were designed according to previously published work (21). The PCR conditions included initial heating to 95°C for 5 mins (denaturation) and followed by 35 cycles of 95°C for 45s, 52°C for 30s, and 72°C for 60s. Final extension was done at 72°C for 7mins. All PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and then sequenced at the DNA Core Facility of Iowa State University using an Applied Biosystems 3730xl DNA analyzer.

Results

Qualitative Test Displayed a Reduction in the Emergence of Ciprofloxacin Resistant Mutants

C. jejuni 11168W199G is a known mutator strain that has a higher mutation frequency for fluoroquinolone resistance (100-fold higher than 11168) (36), which provides an efficient system for measuring the effect of PNA. As shown in Figure 1, there was no visible growth in

the wells treated with either ciprofloxacin or both ciprofloxacin and PNA at 24 hrs of incubation, indicating that ciprofloxacin alone was able to kill most of the *C. jejuni* 11168W199G cells initially. Bacterial growth was obvious in the wells treated with PNA alone, suggesting PNA itself did not kill *C. jejuni*. At 48 hrs of incubation, 60% (10/16) of the ciprofloxacin treated wells showed re-growth due to development of fluoroquinolone-resistant mutants, which was confirmed by growth on MH agar plates with and without ciprofloxacin (1µg/ml). The cultures from the visually clear wells did not show growth on either plate, while the wells that were turbid demonstrated bacterial growth on both MH agar plates with and without ciprofloxacin (1µg/ml). Notably, there was no visible bacterial growth in all the wells co-treated with ciprofloxacin and the CmeA1 PNA, which was further confirmed by plating the cultures on MH agar plates as mentioned above. Half of the wells (4/8) co-treated with ciprofloxacin and the control PNA wells also showed emergence and growth of ciprofloxacin resistant mutants, indicating the control PNA did not potentiate the antibiotic or reduce the emergence of resistant mutants. The wells with visible growth grew on both MH agar plates with and without ciprofloxacin confirming that the growth in the wells was from ciprofloxacin resistant colonies.

The wells treated with PNA (CmeA1 PNA and control PNA) alone showed uniformed growth both at 24 and 48 hrs, and at the end of the experiment when cultures were spread on plates with or without ciprofloxacin (1µg/ml), growth was only observed on the plates without ciprofloxacin, indicating that the culture from the PNA-only treatments remained susceptible to ciprofloxacin. These results indicate that the CmeA1 PNA, not the control PNA, synergized with ciprofloxacin in killing *C. jejuni* and prevented the development of fluoroquinolone-resistant mutants during antibiotic treatment.

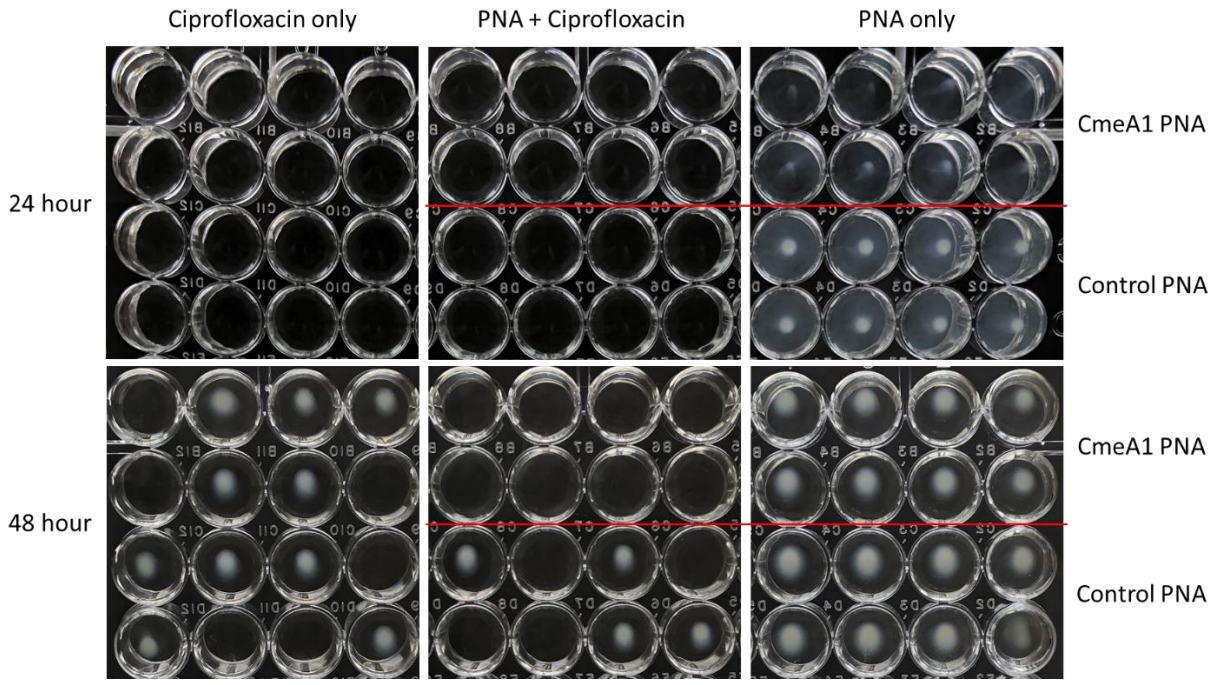

Figure 1. Qualitative test to determine the effectiveness of the CmeA1 PNA at inhibition of the emergence of fluoroquinolone-resistant mutants in 11168W199G.

Quantitative Measurement of Mutant Reduction by PNA

Based on the result described above, we further conducted detailed analysis of mutant emergence by measuring CFU numbers at different time points and the data are shown in Figure 2. At the beginning of the treatment, all treatment groups had similar CFU numbers. At 6 hr, the ciprofloxacin-treated and the co-treated cultures showed significant reduction in CFUs, while the PNA-only treated culture showed growth and remained growing until the end of the experiment. At 12 hr, the ciprofloxacin and PNA co-treated cultures showed no CFU growth and remained this status until the end of the experiment. The CFUs in the ciprofloxacin-only treated cultures continued to decline at 12 hr however it was not as drastic as the CFUs in the ciprofloxacin and PNA co treated cultures. At 24 hr, two of the 3 wells showed no CFU, while 1 of the three showed CFU re-growth, indicating the emergence of resistant mutants in the well. At 48 hr,

however, all three wells of the ciprofloxacin-only treated cultures showed high CFU numbers, indicating the population expansion of ciprofloxacin-resistant mutants.

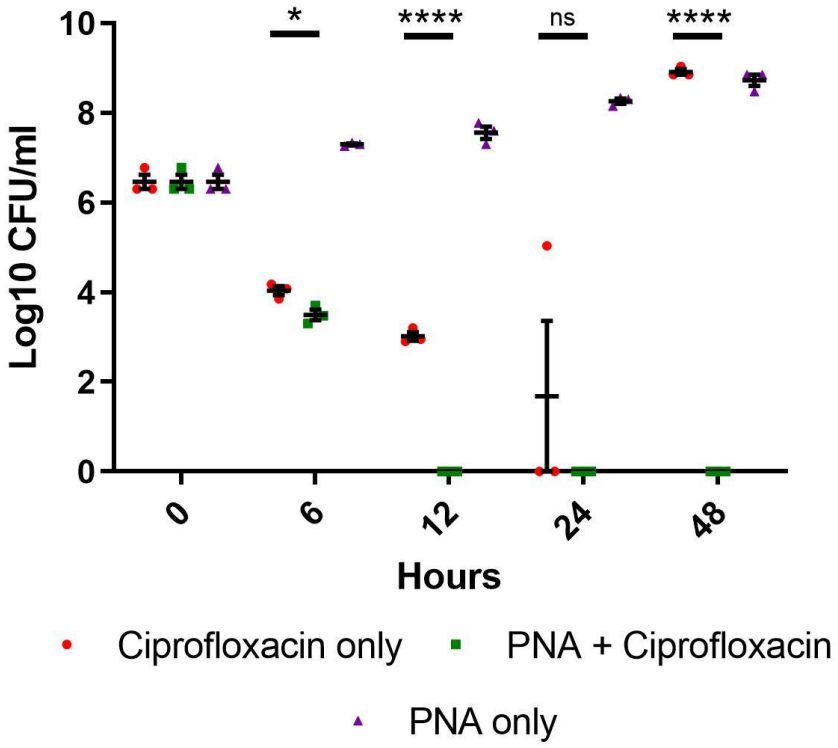

Figure 2. Quantitative determination of CFU counts in cultures with different treatments. The 2 treatment groups (ciprofloxacin only and ciprofloxacin + PNA) were compared at each time point via statistical analysis. * and **** indicate statistically significant difference ($p < 0.05$ and $p < 0.0001$, respectively). ns indicates not significant.

Randomly picked colonies at each time point were assayed for growth or lack of growth on MH plates containing ciprofloxacin (1µg/ml) agar plates to confirm the population change (Table 2). The colonies from the ciprofloxacin-only treated group shifted from susceptible to resistant during the course of the experiment and the shift occurred at the 12-hr time point. The colonies from the ciprofloxacin and PNA co-treated group remained susceptible until all *Campylobacter* cells were killed. Colonies from the PNA-only treated group remained susceptible throughout the experiment. These results correspond with the CFU trends shown in

Figure 2**Error! Reference source not found.** and confirmed the emergence of ciprofloxacin-r esistant mutants in the cultures treated with ciprofloxacin.

Table 2. Growth of randomly picked colonies from each treatment group on MH agar containing ciprofloxacin (1 µg/ml).

Time Points	PNA	PNA + Ciprofloxacin	Ciprofloxacin
0hr	0/5	0/10	0/10
6hr	0/5	0/10	0/10
12hr	0/5	No Colonies	10/10
24hr	0/5	No Colonies	10/10
48hr	0/5	No Colonies	10/10

Quantitative Test Confirmed Elimination of Emergence of Ciprofloxacin Resistant Mutants by PNA

To further confirm the potentiating effect of the CmeA1 PNA on ciprofloxacin, an additional experiment using continuous sampling of the same cultures was performed. Three replicates were used for each treatment and the results are shown in Figure 3. Ciprofloxacin rapidly reduced the CFUs in the treated groups. By 12 hrs, the co-treatment with PNA and ciprofloxacin had eliminated *Campylobacter* from 2/3 of the replicates, while the ciprofloxacin-only treated cultures remained CFUs at a low level. At 24 hrs, the ciprofloxacin-only treated cultures showed re-growth of CFUs, indicating the development and growth of resistant mutants. The CFUs in these cultures continued to increase, and by 48 hrs, the CFU counts are as high as the PNA-only treated cultures. On the contrary, no CFUs were detected in the cultures co-treated with ciprofloxacin and PNA at 24 and 48 hrs. The remaining cultures of this treatment at 48 hrs were further plated onto MH plates containing ciprofloxacin (1µg/ml), and no colonies were

detected, confirming that co-treatment with ciprofloxacin and PNA eliminated the emergence of fluoroquinolone-resistant mutants.

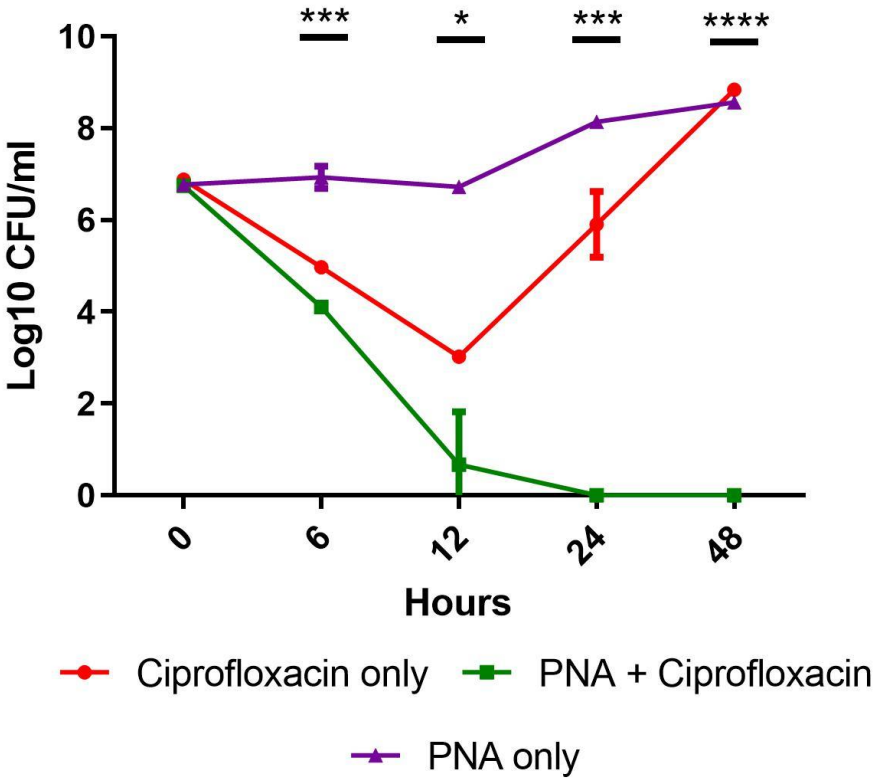

Figure 3. Quantitative measurement of CFUs in cultures of various treatments at different time points. The 2 treatment groups (ciprofloxacin-only and ciprofloxacin plus PNA) were compared at each time point via statistical analysis to determine the differences in the CFU counts. *, *** and **** indicate statistically significant differences ($p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively).

At each time point, a single colony was randomly picked from each replicate from the MH agar plates on which the CFU enumeration was done. These colonies were examined for growth on MH plates containing 1 µg/ml ciprofloxacin. As shown in Table 3, at 0 and 6 hrs, the colonies collected from all treatment groups were susceptible to ciprofloxacin (not able to grow on the plates containing 1 µg/ml ciprofloxacin). At 12 hrs, one colony collected from the PNA and ciprofloxacin co-treated group was susceptible to ciprofloxacin, while 2/3 colonies collected

from the ciprofloxacin-only group were able to grow on the ciprofloxacin-containing plates. At 24 and 48hours, all the colonies collected from the ciprofloxacin-only treated group were resistant to ciprofloxacin, while no colonies were available in the PNA and ciprofloxacin co-treated group for testing as all the *Campylobacter* had been eliminated. The PNA-only treated group had all ciprofloxacin susceptible *Campylobacter* at all time points.

Table 3. Growth of randomly picked colonies from each treatment group on MH agar plates containing ciprofloxacin (1µg/ml).

Time Points	PNA only	PNA + Cipro	Cipro only
0hr	0/3	0/3	0/3
6hr	0/3	0/3	0/3
12hr	0/3	0/2	2/3
24hr	0/3	No Colonies	3/3
48hr	0/3	No Colonies	3/3

Identification of Point Mutations in *gyrA* in Ciprofloxacin-Resistant Mutants.

Ten ciprofloxacin-resistant mutants were randomly selected from ciprofloxacin-only treated group for PCR and sequence analysis. They were amplified by PCR for the QRDR region of the *gyrA* gene and the PCR products were sequenced. All 10 colonies displayed the known G to T point mutation at the 268 position that is associated with fluoroquinolone resistance in *C. jejuni* (36).

Discussion

C. jejuni is known to develop resistance to fluoroquinolone antibiotics swiftly in response to treatment with this class of antibiotics (36, 39). This happens both in culture media and in

animal hosts, and has contributed to the global prevalence of fluoroquinolone-resistant *Campylobacter* (21, 40-44). Given the importance of fluoroquinolones in clinical therapy for campylobacteriosis, it is necessary to develop strategies to prevent the development of fluoroquinolone resistance. In this study, we demonstrated that the CmeA1 PNA specifically targeting the CmeABC efflux pump is able to prevent the emergence of fluoroquinolone resistant mutants in culture media. This conclusion is supported by the results obtained from the qualitative test and the growth kinetics experiments (Figures 1-3) as well as confirmatory testing of randomly selected colonies (Tables 1-3). Additionally, we showed that the CmeA1 PNA enhanced the killing effect of ciprofloxacin as evidenced by the more rapid decline of CFU numbers in the cultures co-treated by ciprofloxacin and PNA compared with the ones treated only with ciprofloxacin. These finding suggest the potential of the CmeA1 PNA as an effective adjunct therapy for clinical use of fluoroquinolone antibiotics.

The use of a mutator strain of 11168 in the experiments allowed us to measure the dynamic changes of resistant mutant development. The qualitative test (Figure 1) not only demonstrated the potentiating effect of the CmeA1 PNA on ciprofloxacin, but also confirmed that this effect is specific for the PNA designed against CmeABC efflux pump (CmeA1 PNA) and was not due to an unspecific side effect of any PNA molecule, because the control PNA was not able to eliminate the emergence of fluoroquinolone resistant mutants. In fact, the treatment with ciprofloxacin and the control PNA yielded similar results to the ones treated by ciprofloxacin only (Figure 1). Therefore, we can conclude that PNA itself did not affect *Campylobacter* growth under the concentration used in this study.

Measuring CFU counts at different time points provided more details on kinetics of resistant mutant development. Ciprofloxacin and PNA co-treatment resulted in continuous

decline of live CFUs until all *Campylobacter* cells were killed, which happened around 12 or 24 hrs after the initiation of the treatment (Figures 2 and 3). On the contrary ciprofloxacin-only treatment produced an initial decline of CFUs, but re-growth was apparent after 12 hrs due to emergence of resistant mutants. It should be pointed out that the emergence of fluoroquinolone-resistant mutants is a spontaneous process, which could explain that only some of the wells/tubes treated with ciprofloxacin developed resistant mutants initially although all the cultures were repopulated by resistant mutants at the end of the experiments (Figures 2 and 3).

The emergence of ciprofloxacin-resistant mutants during the treatments was confirmed by plating the cultures on ciprofloxacin-containing plates and by testing colonies randomly selected at different time points (Tables 2 and 3). Additionally, PCR and sequencing analysis revealed the known mutation in the QRDR of *gyrA* that confers the resistance to fluoroquinolones. It should be mentioned that the 11168W199G strain used in this study has a defective *mutY* and tends to generate G to T mutations (36). In natural isolates, there are other mutations in *gyrA* that also contribute to fluroquinolone resistance such as the C257T mutation (20). In either case, GyrA mutation alone are not sufficient to confer high-level resistance to fluoroquinolones. These mutations work synergistically with the CmeABC efflux pump to achieve high MICs of ciprofloxacin (26, 38, 41).

Small molecule inhibitors, such as Efflux Pump Inhibitors (EPIs), can be used to interfere with the activity of the efflux pump; however, these are known to be inconsistent with their inhibition capabilities (45-47). Furthermore, the concentrations required for inhibition are highly toxic *in vivo* (48). PNA on the other hand has already been used in mammalian studies and did not show any toxic effects (49, 50). This characteristic combined with the high specificity of target inhibition and resistance to enzymatic degradation makes PNA a promising candidate for

an adjunct therapeutic. As shown in three independent experiments (Figures 1-3), the CmeA1 PNA was able to prevent the emergence of fluoroquinolone resistant mutants. Since CmeABC is known to work in synergy with other resistance mechanisms to confer high MICs (11, 17, 24), it is reasonable to say that inhibiting CmeABC by the CmeA1 PNA would increase the susceptibility of the resistant mutants to ciprofloxacin and leads to complete killing of the mutants by the antibiotic. Altogether, the results from this study demonstrate the potentiating effect of the CmeA1 PNA on ciprofloxacin against *C. jejuni* and provide a strong rationale for conducting *in vivo* studies to assess the efficacy of the PNA as an adjunct therapy.

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CHAPTER 4. EFFECT OF ANTI-CmeABC PEPTIDE NUCLEIC ACID ON THE EMERGENCE OF FLUOROOQUINOLONE-RESISTANT *CAMPYLOBACTER* IN CHICKENS

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Abstract

Campylobacter jejuni is the leading cause of bacterial gastroenteritis worldwide and is increasingly resistant to fluoroquinolone (FQ), which are clinically used antibiotics for the treatment of campylobacteriosis. FQ resistance spontaneously occur in *C. jejuni* and our previous work showed that antisense peptide nucleic acid (PNA) targeting the CmeABC efflux pump prevented the emergence of FQ-resistant mutants in *C. jejuni* when treated with ciprofloxacin in culture media. However, the efficacy of the PNA in potentiating the antibiotic under in vivo conditions has not been evaluated. In this study, chickens infected by FQ-susceptible *C. jejuni* were subjected to treatment with enrofloxacin (50 ppm in drinking water) with or without the PNA administered via oral gavage. In three independent trials, FQ-resistant *Campylobacter* consistently emerged in the chickens treated with enrofloxacin, and the PNA, when given at a dose of 2 μM per bird, initially reduced the development of FQ-resistant mutants but did not eliminate the mutants in the birds treated with enrofloxacin. Doubling the daily dosing of PNA did not further increase the efficacy of the PNA. PNA alone, when given at the same dose, did not show apparent toxicity to the birds and did not affect *Campylobacter* colonization and development of FQ-resistant mutants in chickens. These results demonstrate the potential of the PNA as an adjunct therapy to potentiate FQ antibiotics in reducing the development of FQ-

resistant *Campylobacter*. Further studies are needed to optimize the dose and treatment scheme of antibiotic and PNA to eliminate the emergence of FQ-resistant mutants.

Introduction

Campylobacter jejuni is a gram negative, curved rod shaped, and thermophilic bacterium. It colonizes the intestinal mucosa of most warm-blooded animals, including all food-producing animals and the human host. *C. jejuni* is especially prevalent in avians, such as chickens, turkeys, quails, ducks, and wild birds (1). In these animals, *C. jejuni* is generally considered a commensal, but in humans it causes acute gasteroenteritis (2, 3). In fact, *C. jejuni* is one of the most common causes of bacterial food-borne disease. According to the CDC, there are over 1.3 million cases of campylobacteriosis ever year in the United States (4). Worldwide, *Campylobacter* infections are estimated to be responsible for 400-500 million cases of diarrhea each year (5). *C. jejuni* is known to be able to survive the poultry meat processing and throughout the food supply chain, which constitutes a major source of infection for sporadic cases of campylobacteriosis in humans. On the other hand, consumption of raw milk is a main risk factor for outbreaks of human *Campylobacter* infections (3, 6).

For clinical treatment of human campylobacteriosis, fluoroquinolones and macrolides are the first choice of antibiotics (7, 8). Unfortunately, *Campylobacter* has become increasingly resistant to clinically important antimicrobials over the years (9-11). The World Health Organization (WHO) has listed antibiotic resistant *Campylobacter* as a pathogen of high concern that urgently needs novel therapeutic strategies to combat infection (12). *Campylobacter* has developed multiple mechanisms for antibiotic resistance, such as target modification by acquiring point mutations and target protection by acquiring resistance genes through horizontal

gene transfer (e.g *tetO* that confers tetracycline resistance) (10, 11). A major mechanism for multi-drug resistance in *Campylobacter* is the CmeABC efflux pump (13).

The CmeABC efflux pump belongs to the resistance-nodulation-division (RND) family of membrane transporters and is used by *Campylobacter* to extrude toxic compounds, such as bile and antimicrobials (14). CmeABC works synergistically with other resistance mechanisms and confers resistance to structurally diverse antibiotics. It has been shown that CmeABC increases the emergence of spontaneous fluoroquinolone-resistant mutants in *Campylobacter* as inactivation of the *cmeABC* operon led to significant decrease in the detected mutant frequency (15). Owing to its key function in bile resistance, CmeABC is essential for *Campylobacter* colonization in the intestinal tract of an animal host (14). Given the key role of CmeABC in antimicrobial resistance and gut colonization, inhibition of this efflux pump is a promising strategy for combating antibiotic-resistant *Campylobacter* (16).

Campylobacter is a zoonotic pathogen and is therefore exposed to antibiotics used in both animal production and human medicine. Ciprofloxacin is a fluoroquinolone antibiotic and is commonly used to treat bacterial infections in human patients. Fluoroquinolones were also used on poultry farms (for treatment of other bacterial infections such as colibacillosis) until 2005, when they were banned in the United States (17). The decision to ban fluroquinolones on poultry farms was incited due to the drastic rise of fluoroquinolone resistant *Campylobacter* since the use of the antibiotic in poultry (18, 19). This had raised a major public health concern as fluoroquinolone resistant *Campylobacter* can be transmitted to humans via contaminated retail poultry meat, resulting in campylobacteriosis that is less receptive to fluoroquinolone therapy (20). Multiple studies have shown that the use of fluoroquinolones in broiler chickens selects fluoroquinolone-resistant *Campylobacter* from originally fluoroquinolone-susceptible

Campylobacter, and fluoroquinolone-resistant *Campylobacter* rapidly appeared during the treatment, resulting in replacement of the susceptible *Campylobacter* population in the treated birds (21, 22). Since the emergence of fluoroquinolone resistance from susceptible *Campylobacter* in chickens treated with enrofloxacin is well documented, chicken is an ideal animal model to test if inhibition of the CmeABC efflux pump reduces the emergence of fluoroquinolone resistance.

In general, there are two ways to inhibit bacterial efflux: blocking its function or inhibiting its expression (23, 24). Efflux pump inhibitors (EPIs) are molecules that specifically block the efflux function of MDR efflux pumps. This can be accomplished by physically obstructing the efflux pump channel or by competing with the natural substrates. EPIs could also inhibit efflux activity by altering the pump assembly or destroying its energy mechanism (25). EPIs have been attempted to inhibit the CmeABC efflux pump in *Campylobacter*. Phenyl-arginine-β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) are 2 EPIs that have been used to lower the minimum inhibitory concentrations (MICs) of fluoroquinolones and macrolides (26-28). The results from different studies varied greatly and were not consistent. Furthermore, none of these EPIs have been tested *in vivo* as the previous studies were conducted only in culture media. Additionally, EPIs are generally toxic at the levels required for inhibition and many modifications made to the structures to reduce their toxicity also resulted in loss of inhibitory effects on efflux pumps and reduce stability in animal serum (24, 29-33). Thus, safe and efficacious EPIs are yet to be developed as an adjunct therapy for antibiotic therapy.

Peptide nucleic acids (PNA) on the other hand can be designed to inhibit the expression of efflux pumps instead of blocking its function. PNAs are synthetic polymers, composed of N-(2-aminoethyl) glycine units with nucleobases attached to the glycine nitrogen via carbonyl

methylene linkers, and have been successfully used as a drug in mammals without any toxic effects (34, 35). PNA has the ability to base pair specifically with DNA and RNA due to the nucleobases in the composition that mimic nucleic acid structure (36, 37). This characteristic allows PNA to target expression of a specific sequence with antisense inhibition (38). It is also resistant to enzymatic degradation and remains in tissues for long periods of time (39, 40). Therefore, it is a promising molecule that can be designed to inhibit expression of genes conferring antimicrobial resistance. Previously, it was shown that PNA-mediated antisense translational inhibition of the TEM-1 β-lactamase re-sensitize drug-resistant *Escherichia coli* to β-lactam antibiotics (41). PNA was also designed to inhibit the expression of the CmeABC efflux pump (42). The CmeA1 PNA (anti-CmeA PNA) was able to reduce the production of CmeA as confirmed by western blots and was successful at increasing the susceptibility of *Campylobacter* to ciprofloxacin and erythromycin (16). We have also established that the CmeA1 PNA is able to reduce the emergence of ciprofloxacin resistant mutants under selection pressure *in vitro* (Chapter 3). Despite these advances, the *in vivo* efficacy of PNA in inhibiting CmeABC and sensitizing *Campylobacter* to antibiotics has not been examined. To close this knowledge gap, we conducted *in vivo* treatment studies using chicken as an animal model.

Materials and Methods

Bacterial Strains and Growth Conditions

C. jejuni strains were cultured microaerobically in a gas chamber filled with 5% O_2, 10% CO_2 and 85% N_2 on Mueller-Hinton (MH) agar or in Mueller-Hinton (MH) broth at 42 °C. All strains were preserved as 30% glycerol stock at -80°C. Two strains were used in this study, NCTC 11168 and CT6:16L (ciprofloxacin MIC of both strains = 0.063 µg/ml). CT6:16L is a *C.*

jejuni isolate derived from commercial turkeys (43). Both strains were confirmed to be motile prior to inoculation into the chickens.

Antimicrobial Susceptibility Tests

Ciprofloxacin used to perform antimicrobial susceptibility tests in the laboratory was purchased from Sigma-Aldrich Co. LLC. The MIC was determined using the standard broth microdilution method in MH broth with an inoculum of 10^6 CFU/ml as previously described (44). Round bottomed 96 well culture plates were used, which were incubated for 24 hours under microaerophilic conditions at 42 °C. *C. jejuni* NCTC 11168 was used as a quality control for the test.

PNA Preparation

Based on the results from previous work.(42), the Peptide Nucleic Acid (PNA) CmeA1 that binds to the translational start region of *cmeA* was synthesized and conjugated with a cell penetrating peptide (CPP) by PNA BIO INC (Newbury Park, CA). The CmeA1 sequence is as follows: KFFKFFKFFK-tgccttgaaaaa. The PNA was received as a lyophilized powder that was then reconstituted using autoclaved dd.H_2O to a stock concentration of 2,000 µM or 4,000 µM, aliquoted into vials, and stored at -20°C for use.

Antibiotic Treatment via Drinking Water

Enrofloxacin (Sigma-Aldrich Co. LLC., USA) was administered to chickens via drinking water as described previously (21). The antibiotic was dissolved in DMSO to make a 10 mg/ml solution, 5 ml of which was added to 1L of tap water to make a drinking solution with 50 ppm of enrofloxacin. The drinking water for the non-treated group of chickens did not have antibiotic but had the same concentration of DMSO diluted in tap water (5 ml in 1L). Fresh drinking water solutions were prepared each day and given to the chickens during the treatment period.

Collection of Cloacal Swabs and Cecal Contents

Cloacal swabs and cecal contents were collected during the chicken experiments to determine *C. jejuni* colonization levels and emergence of fluoroquinolone-resistant *Campylobacter* in the intestinal tract. Sterile cotton swabs were used to collect approximately 100 mg feces from each chicken. The individual swabs were placed in sterile tubes with 1ml MH broth until they were processed. Cecal contents were collected from 1 or both ceca after euthanasia and necropsy of the chickens. Approximately 500 mg feces was collected from each bird and kept on ice until processed.

***Campylobacter* CFU Enumeration**

The cloacal swabs were diluted in MH broth with 10-fold dilution series. For each dilution, 100 µl was plated onto a MH agar plate. To culture *Campylobacter* from feces, *Campylobacter* growth supplements and *Campylobacter* selective supplements (Oxoid) were added to the MH media to reduce the growth of background bacterial flora. The plates were incubated at 42 °C microaerobically for 48 hours for total CFU counts of *Campylobacter*. In parallel, the fecal dilutions were also plated onto *Campylobacter* selective MH agar plates with ciprofloxacin (4 µg/ml) to obtain fluoroquinolone-resistant *Campylobacter* CFU per gram of feces. The CFU/g of feces was calculated based on the colony counts and dilution factors. For the cecal contents, the amount of feces was first weighed, and then 10-fold dilution series were made in MH broth. Culturing methods and conditions as well as calculation of CFU/gram of feces were done in the same as cloacal swabs mentioned above.

Statistical Analysis

An unpaired student's t test was used to compare the average *Campylobacter* CFU/g of feces between different treatment groups in the chicken experiments. P values of < 0.05 were deemed to be statistically significant. Graphpad prism 8 software was used to generate the graphs.

Design of Chicken Trial 1

Day-old layer (white leghorn) chickens were obtained from a commercial hatchery. The chickens were randomly divided into 5 treatment groups, each group was housed in a dedicated wire floored brooder to avoid cross contamination between groups. Treatment groups are listed below in Table .

Table 1. Treatment groups for Trial 1.

Group	Treatment	No. of chicks
1	Enrofloxacin (50ppm)	6
2	Enrofloxacin (50ppm) + PNA (0.2 µmoles/day)	5
3	Enrofloxacin (50ppm) + PNA (2 µmoles/day)	5
4	PNA (2 µmoles/day)	5
5	No Treatment	5

Prior to inoculation, the birds were tested negative for *Campylobacter* by culturing cloacal swabs as detailed above. At 5 days of age, each chicken was inoculated with 200 µl of 10^6 CFU/ml *C. jejuni* 11168 (Cipro MIC 0.063 µg/ml) via oral gavage. Five days post inoculation (DPI), cloacal swabs were collected to confirm *Campylobacter* colonization. Feed

and water were provided ad libitum. On DPI 7, treatment was initiated in drinking water. Groups 1-4 received enrofloxacin or PNA or both (Table 1), while group 5 was given regular water with DMSO. Enrofloxacin was administered in drinking water, while PNA was given via oral gavage (once a day). The treatment lasted for 5 days, from DPI 7 to DPI 11. Cloacal swabs were collected periodically for culturing *Campylobacter*. On DPI 16, all chickens were euthanized and cecal contents were collected for quantitative enumeration of *Campylobacter* CFUs.

Design of Chicken Trial 2

Based on the results of trial 1, we modified the treatment and sampling schemes in trial 2. Day-old layer (white leghorn) chickens were obtained from a commercial hatchery. The chickens were divided into 4 treatment groups as listed in Table 2. Prior to inoculation, the birds were tested negative for *Campylobacter* by culturing cloacal swabs as detailed above. At 4 days of age each chicken was inoculated with 200 µl of 10^7 CFU/ml *C. jejuni* CT6:16L (Cipro MIC = 0.063µg/ml) via oral gavage. On DPI 5, cloacal swabs were collected to confirm *Campylobacter* colonization. Feed and water were provided ad libitum.

Table 2. Treatment groups for Trial 2.

Group	Treatment	No. of chicks
1	Enrofloxacin (50ppm)	14
2	Enrofloxacin (50ppm) + PNA (2 µmoles/day)	12
3	PNA (2 µmoles/day)	6
4	No Treatment	8

On DPI 7, treatment was started. Based on the result of trial 1, the lower PNA dose (0.2 µmoles per day) was not used because it was not effective. The treatments were conducted in the same manner as described for trial 1 and lasted for 5 days, from DPI 7 to DPI 11. Cloacal swabs were collected on DPI 8, 24 hours after the first PNA treatment. On DPI 9 (after 2 days of treatment), 4 chickens each from group 1 and 2 were euthanized and cecal contents were collected, while 3 chickens each from group 3 and 4 were terminated by euthanasia for collection of cecal contents. On DPI 11 (after 4 days of treatment), 4 chicken from each of groups 1 and 2 were necropsied for collection of cecal contents. All the remaining chickens were euthanized on DPI 12 (1 day after the end of treatment) and their cecal contents were collected for culturing *Campylobacter*.

Design of Chicken Trial 3

A major change in trial 3 was to increase the dosing frequency of PNA to determine whether it provided better potentiating effect on enrofloxacin. The same dosage (2 µmoles) of PNA was given to each chicken twice a day instead of once a day, and the treatment was given for 4 days. Again, 1 day-old layer (white leghorn) chickens were obtained from a commercial hatchery and were divided into 4 treatment groups as listed in Table 3. As was the case with previous trials, cloacal swabs were collected to confirm the birds were free of *Campylobacter* prior to inoculation. Each bird was inoculated with 200 µl of 10^6 CFU/ml *C. jejuni* CT6:16L (Cipro MIC 0.063µg/ml) via oral gavage at 8 days of age. On DPI 5, cloacal swabs were collected to confirm *Campylobacter* colonization. Feed and water were provided ad libitum.

Table 3. Treatment groups for Trial 3.

Group	Treatment	No. of chicks
1	Enrofloxacin (50ppm)	10
2	Enrofloxacin (50ppm) + PNA (2 µmoles twice a day)	10
3	PNA (2 µmoles twice a day)	6
4	No Treatment	4

On DPI 7, treatment was initiated (see Table 3 for treatment scheme) and the treatment lasted for 4 days, from DPI 7 to DPI 10. Cloacal swabs were collected on DPI 7 before the start of treatment and on DPI 8 after 24hours of first treatment. On DPI 9 (after 2 days of treatment), 5 chickens each from groups 1 and 2 were euthanized and cecal contents were collected for culturing *Campylobacter*. Also, 3 chickens from group 3 and 2 chickens from group 4 were euthanized for necropsy and cecal contents. On DPI 11(one day after the end of treatment), all remaining chickens were euthanized for collection of cecal contents.

Results

Determination of PNA Doses

As seen in Figure 1, by DPI 5 the chickens were all colonized with *Campylobacter*. Before initiation of the treatment with enrofloxacin, all the chickens had ciprofloxacin susceptible *Campylobacter* (Fig. 1B and 1D). The CFU counts dropped on DPI 8 (24 hours after initiation of treatment) in the groups receiving enrofloxacin treatment. In the group that only received enrofloxacin, the CFU counts rapidly rebounded due to emergence of resistant mutants as the treatment progressed, consistent with the results from previous treatment experiments in chickens

(21, 22, 45). The groups co-treated with the low-dose PNA did not appear to affect the development of ciprofloxacin-resistant mutants (Fig. 1 A and 1B); however, the group that was co-treated with the high dose of PNA consistently showed lower CFU counts (Figure 1C and 1D), indicating that PNA reduced the emergence of resistant *Campylobacter* when PNA was given together with enrofloxacin. Although there was a clear trend of reduction in CFU counts in the high dose PNA co-treated group, the differences were not statistically significant, probably due to variability between animals and the small number of animals in each group. Regardless, the results indicate that the low dose of PNA (0.2 µmoles/bird/day) is not sufficient to potentiate the effect of antibiotic treatment. There were no significant differences in colonization levels of the 2 control groups: PNA only and No Treatment (Figure 1E), indicating that PNA alone did not affect *Campylobacter* colonization in chickens. Additionally, no Ciprofloxacin resistant colonies were detected in the control groups during the entire course of experiment (Figure 1F).

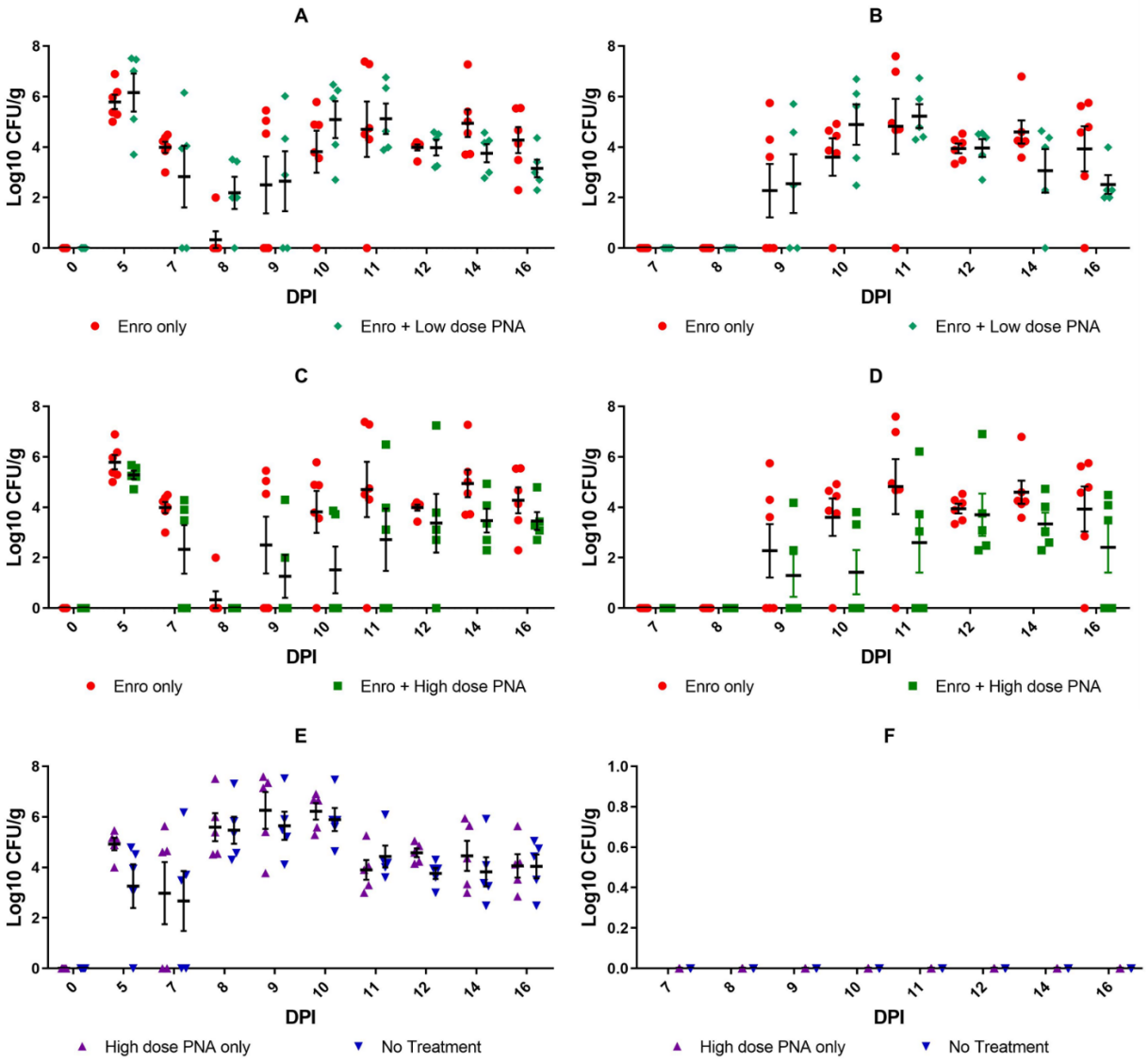

Figure 1. CFU enumeration of *C. jejuni* 11168 from chicken cloacal swabs. (A) Comparison of total CFU counts in chickens from 2 treatment groups: Enro only and Enro + low dose PNA. (B) Comparison of FQ-resistant CFU counts in chickens from 2 treatment groups: Enro only and Enro + low dose PNA. (C) Comparison of total CFU counts in chickens from 2 treatment groups: Enro only and Enro + high dose PNA. (D) Comparison of FQ-resistant CFU counts in chickens from 2 treatment groups Enro only and Enro + high dose PNA. (E) Comparison of total CFU counts in chickens from 2 control groups: High dose PNA only and No treatment. (F) Comparison of FQ-resistant CFU counts in chickens from 2 control groups: High dose PNA only and No treatment.

Effect of PNA on a Different *C. jejuni* Strain

Similar to *C. jejuni* 11168, *C. jejuni* CT6:16L was able to colonize chickens by 5 days post inoculation (Fig. 2A). All the chickens were colonized by ciprofloxacin susceptible *Campylobacter* before the initiation of treatment (Figure 2C). CFU counts in cloacal swabs significantly decreased on DPI 8 (24 hours after initiation of treatment) in the groups receiving enrofloxacin treatment. However, ciprofloxacin-resistant *Campylobacter* emerged rapidly in the groups treated with enrofloxacin. Compared to the group treated with enrofloxacin only, the group that was co-treated with PNA displayed reduced CFU counts of ciprofloxacin-resistant *Campylobacter*, and the difference was statistically significant on DPI 11 (Figure 2B and 2D). Different from experiment 1 (Fig. 1), in which CFU counts were obtained from cloacal swabs, cecal contents derived from necropsy were used to enumerate the *Campylobacter* CFUs after initiation of the treatment. Use of cecal contents for CFU counts reduced variability between animals within a group. After the termination of treatment (DPI 12), there was no longer significant difference in the CFU counts between the 2 treatment groups (enrofloxacin alone and enrofloxacin plus PNA) (Fig. 2B and 2D). In the control groups (PNA only and no treatment), no ciprofloxacin-resistant *Campylobacter* was detected during the experiment (Figure 2C and 2D). Overall, the results indicate that co-treatment with PNA reduces but does not eliminate the emergence of fluoroquinolone resistant mutants during treatment with fluoroquinolone antibiotics.

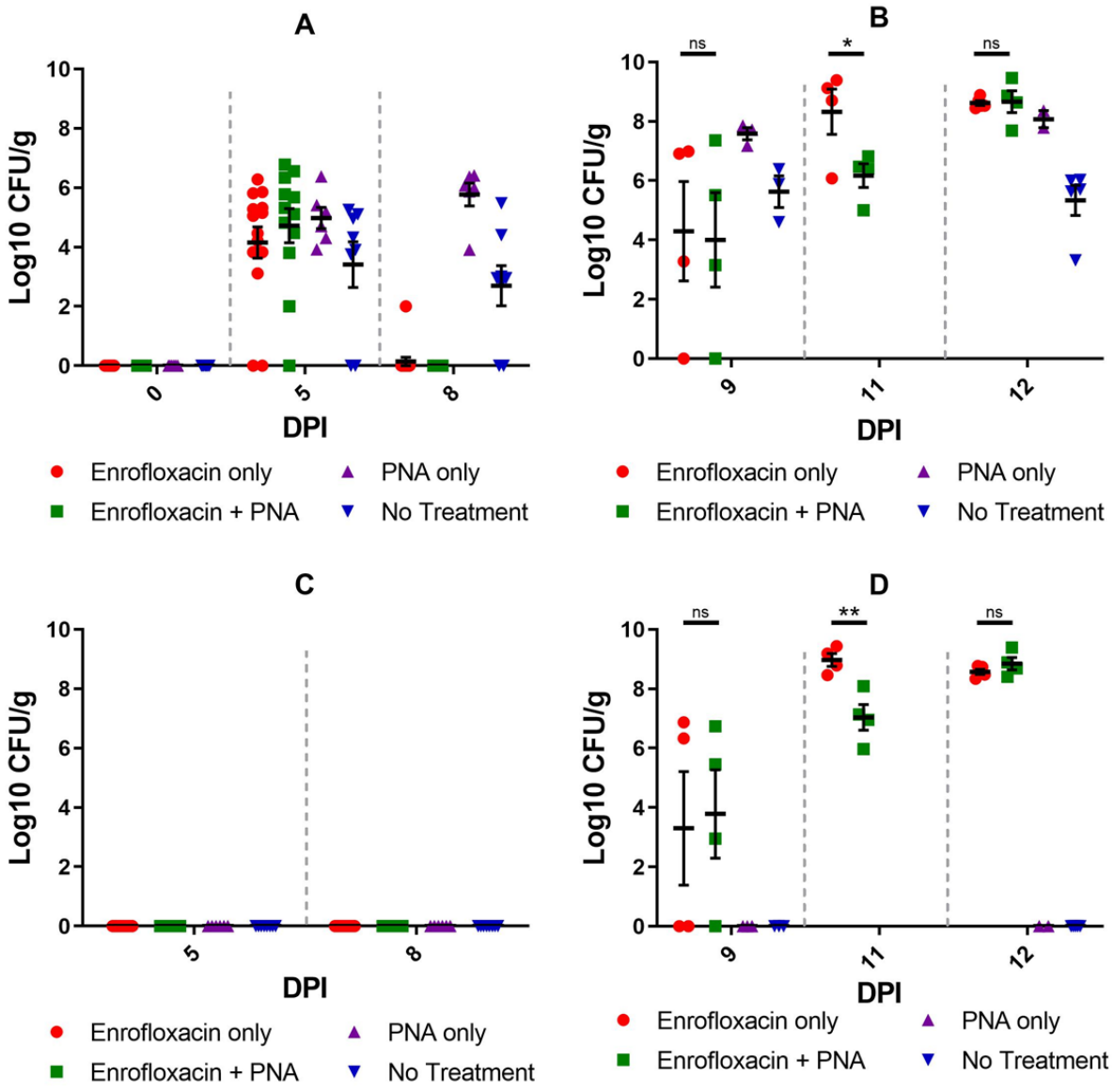

Figure 2. CFU enumeration of *C. jejuni* CT6:16L from chicken cloacal swabs and cecal contents. (A) Total CFU counts in chicken cloacal swabs from DPI 0 to 8. (B) Total CFU counts in chicken cecal contents from DPI 9-12. (C) Fluoroquinolone-resistant CFU counts in chicken cloacal swabs from DPI 5-8. (D) Fluoroquinolone-resistant CFU counts in chicken cecal contents from DPI 9-12. * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively). ns indicates not statistically significant.

Effect of PNA Dosing Frequencies

Similar to the results from Trial 1 and Trial 2, the chickens were colonized by *C. jejuni* by DPI 5 as determined by culturing cloacal swabs (Figure 3A). Once treatment with enrofloxacin was initiated, the CFU counts dropped drastically, but resistant mutants emerged quickly in the treated birds (Fig, 3B, 3C, and 3D). The group co-treated with PNA had lower CFU counts than the group treated with enrofloxacin only, and the reduction in total CFU was statistically significant on DPI 9 (Fig. 3B). After the termination of treatment there was no longer difference in the CFU counts between the 2 treatment groups. Ciprofloxacin CFU counts on DPI 9 revealed a trend of reduction in the PNA co-treated group, but the difference was not statistically significant (Fig. 3D), Together, the results suggest that increasing dosing frequency of PNA (twice per day) did not further enhance its potentiating effect on enrofloxacin.

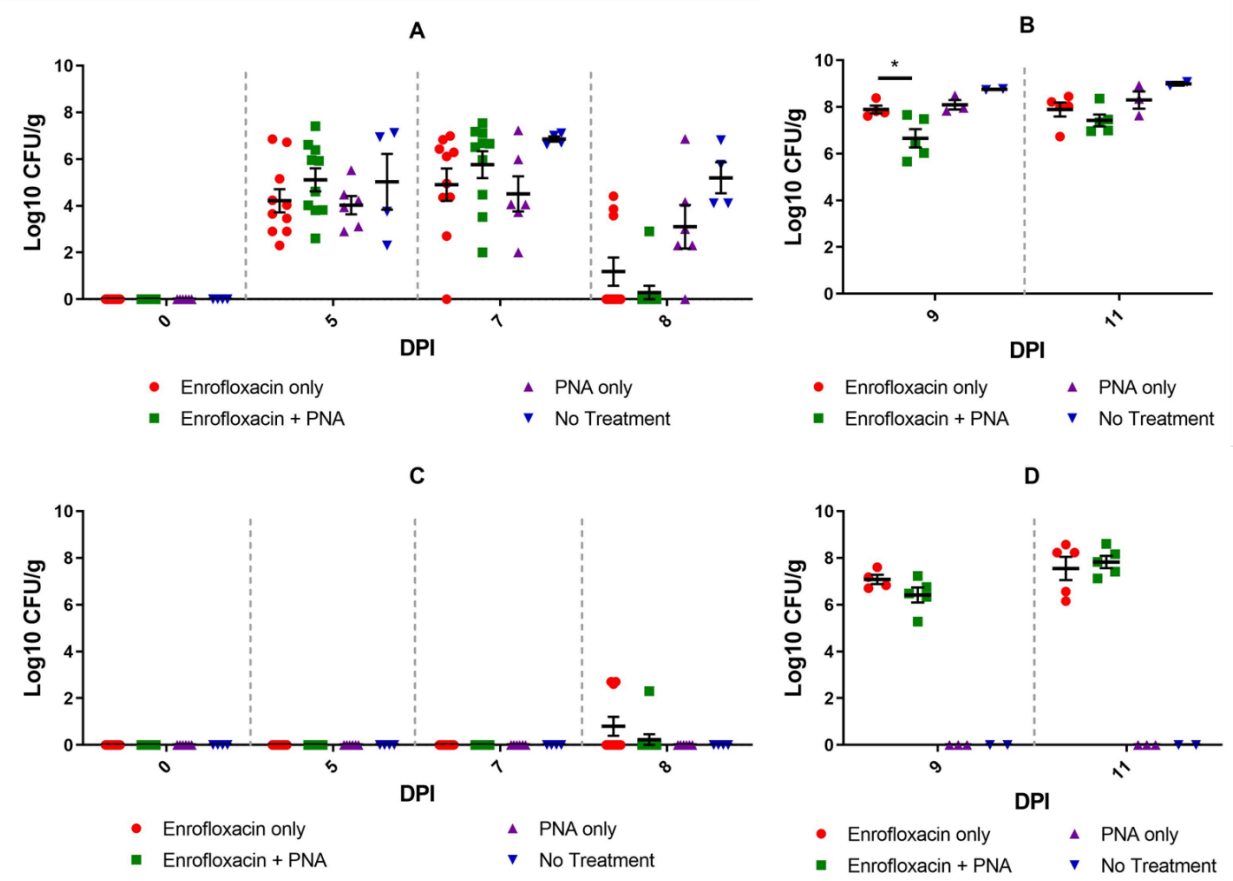

Figure 3. CFU enumeration of *C. jejuni* CT6:16L in chicken cloacal swabs and cecal contents. (A) Total CFU counts in chicken cloacal swabs from DPI 0-8. (B) Total CFU counts from chicken cecal contents from DPI 9-11. (C) Cipro resistant CFU counts in chicken cloacal swabs from DPI 0-8. (D) Cipro resistant CFU counts in chicken cecal contents from DPI 9-11. *indicates statistically significant difference ($p < 0.05$).

Confirmation of Ciprofloxacin Resistance

In addition to measuring ciprofloxacin resistant CFUs by use of differential plating, we also picked single colonies for confirmation of their susceptibility to ciprofloxacin. A single colony was randomly collected from the non-antibiotic plate of each sample at each sampling time and subsequently purified by subculture. All the collected isolates were identified using MALDI-TOF MS to confirm that they were *C. jejuni*. All the isolates were first examined on MH agar plates containing ciprofloxacin (4 µg/ml). The colonies isolated from the control groups (PNA only and No treatment) of the three trials were always not able to grow on the

plates, indicating they were susceptible to ciprofloxacin. In all 3 trials, all isolates collected from enrofloxacin treatment groups prior to the initiation of the treatment were susceptible to ciprofloxacin.

The isolates picked on DPI 8, one day after the initiation of treatment, were still susceptible and this was likely that resistant mutants just started to emerge and remained low in number in the treated birds. On DPI 9 (2 days after initiation of the treatment), 6 out of the 8 colonies picked from Trial 1 were resistant to ciprofloxacin, 5 out of the 6 colonies picked from Trial 2 were resistant to ciprofloxacin and in Trial 3 all 9 colonies that were picked were resistant to ciprofloxacin. This indicates that the majority of the colonies (75% for trial 1, 83% for trial 2, and 100% for trial 3) were resistant to ciprofloxacin by DPI 9 as determined by growth on plates containing 4 µg/ml of ciprofloxacin. From DPI 10 onwards, all the colonies picked from the chickens in the enrofloxacin-treated groups were resistant to ciprofloxacin. Furthermore, 1 randomly selected isolate from the enrofloxacin-treated groups at each sampling day were subjected to antimicrobial susceptibility tests for MICs of ciprofloxacin. All susceptible isolates tested had an MIC of 0.063 µg/ml, while the resistant isolates had an MIC of 16 µg/ml. These results are consistent with the differential plating results and confirm the emergence of ciprofloxacin-resistant *Campylobacter* in enrofloxacin-treated birds.

Discussion

Result from this study exhibited that ciprofloxacin-resistant *Campylobacter* developed rapidly in chickens treated with enrofloxacin, and the PNA specifically targeting CmeABC efflux pump was able to reduce the emergence of fluoroquinolone resistant mutants *in vivo*. The potentiating effect of PNA was seen in the early stage of the treatment but was abolished as the treatment terminated. This effect was observed in all three trials (Figure 1, 2, and 3) and with 2

different strains of *C. jejuni*, These findings demonstrate the potential use of the anti-CmeABC PNA for *in vivo* treatment, but further studies are needed to optimize the dosages and delivery as the PNA did not eliminate the emergence of ciprofloxacin-resistant *Campylobacter* in this study.

Trial 1 was performed using a laboratory strain *C. jejuni* NCTC 11168 as it was previously shown to be able to colonize chickens and ciprofloxacin-resistant mutants readily emerged in the chickens upon treatment with enrofloxacin (21, 22, 45). Since no previous in vivo studies have been conducted with an anti-*cmeABC* PNA delivered orally, we tested 2 different doses of PNA in the first trial. The doses were chosen based on the *in vitro* results and the expected complexity in the gastrointestinal contents. The results demonstrated that the lower dose (0.2 µmoles/bird/day) did not influence the emergence of ciprofloxacin-resistant mutants in enrofloxacin-treated birds (Figure 1A&B), while the high dose (2 µmoles/bird/day) appeared to reduce the emergence of fluoroquinolone resistant mutants. When analyzed statistically, the difference was not significant, probably due to the fact that cloacal swabs were used for CFU determination in trial 1, and it has been known that CFU counts tend to vary greatly even among birds inoculated with the same strain of *C. jejuni*.

In trial 1, all of the birds in a group were housed within a single brooder, which means between-host transmission occurred within the same group. It is likely that even if ciprofloxacin-resistant mutants developed in one bird, they would likely had transmitted to the other chickens in the same brooder. This can be seen in Figure 1D, on DPI9 and DPI10, only 2 out of the 5 chickens in the enrofloxacin and PNA co-treated group developed ciprofloxacin resistant mutants, while in the group treated with enrofloxacin only, 3 out of 6 chickens developed ciprofloxacin resistant colonies on DPI9 and 5 out of 6 chickens on DPI10. On DPI 11, however, 3 out of 5 chickens in the PNA co-treated group had resistant *Campylobacter* and on DPI 12 all 5

chickens were colonized with ciprofloxacin resistant *Campylobacter*. This indicates that the CmeA1 PNA was initially able to reduce the emergence of resistant mutants but was unable to completely inhibit the development of the mutants.

Cecum is the primary site for *Campylobacter* colonization in chickens and CFU counts in cecal contents are better indicators for the level of colonization compared to the CFUs from cloacal swabs. Thus, in trial 2, birds were sacrificed after initiation of the treatment for collecting cecal contents, and total *Campylobacter* CFUs and ciprofloxacin-resistant CFUs were determined. Cloacal swabs were only collected initially to confirm colonization before treatment was started because of the need for sacrificing birds at different time points, the number of birds required for the treated groups were larger. Thus, in the 2nd trial, the birds in each of groups 1 and 2 (Table 2) were divided into 4 different brooders. This was also expected to help limit between-bird transmission within the same group. The results of trial 2 showed the same trend as trial 1, but both total CFUs and ciprofloxacin-resistant CFUs were significantly reduced on DPI 11 in the enrofloxacin and PNA co-treated group compared to the group treated with ciprofloxacin only (Figure 2). However, the colonization levels in the two groups became similar by the end of the treatment. These results further indicated that PNA was able to reduce but was unable to eliminate the emergence of fluoroquinolone resistant mutants *in vivo*.

Based on the results of trials 1 and 2, we hypothesized that increasing dosing frequency of PNA might be able to enhance its potentiating effect on enrofloxacin. Thus, in trial 3, PNA was administered twice a day with a 12 hours interval. The results as seen in Figure 3, however, is much similar to the result of trial 2 (Figure 2), indicating that giving 2 doses a day did not further increase the efficacy of the PNA treatment. The result suggests that a higher dose of PNA

or improved delivery is needed to improve its efficacy in potentiating fluoroquinolone antibiotics.

In the *in vitro* experiments using culture media, we were able to demonstrate that ciprofloxacin and CmeA1 PNA co-treatment completely eliminated the emergence of ciprofloxacin resistant mutants (chapter 3). This indicates the efficacy of the PNA and suggests that if present in sufficient concentration, the PNA should be able to completely inhibit the emergence of ciprofloxacin resistant mutants. However, the conditions in animal intestine are much more complex, and there are many variables and factors that may influence the efficacy of PNA in animal models. For example, *Campylobacter* only accounts for a tiny portion of the gut microbiota and the presence of other bacterial flora may significantly reduce the availability of PNA to Campylobacter. Additionally, the concentration of PNA in the gut cannot be controlled accurately due to gradient of concentrations existing in the gut and variable frequencies of defecation. Thus, the effective concentration of PNA in the ceca, where *Campylobacter* colonize, likely vary greatly from animal to animal. The stability of PNA in the intestinal tract is also unknown. In a previous study, rats were injected with a PNA intravenously, and approximately 90% of the total PNA given was recovered from the urine 24 hours after administration (40). Another study administered fluorescent labeled PNA intraperitoneally in mice once per week for 3 weeks and they obtained good target inhibition (34). Unlike these studies, the CmeA1 PNA was administered orally and was exposed to the harsh environments in the gastrointestinal tract. All these factors discussed above may influence the efficacy of PNA and contribute to the variable effects in individual animals.

Although PNA has been used against bacterial gene expression *in vitro* (41, 46, 47), its effectiveness in reducing bacterial load in animal models is rarely tested. In a study conducted in

BALB/c mice injected with *E. coli* to induce bacteremia and peritonitis, a PNA targeting an essential gene (*acpP*) was administered 30 mins before challenge intraperitoneally (48). This resulted in a significant reduction of bacterial load in the blood. In the next set of experiments, they administered PNA intravenously 30 mins after challenge and again observed a significant reduction in bacterial load (48). Another study was conducted in the *Galleria mellonella* model of sepsis caused by Multidrug-resistant (MDR) *Acinetobacter baumannii*. Two doses (5 μM and 20 μM) of PNA targeting an essential gene (*carA*) were evaluated by administering 30 mins post inoculation. Although the low dose of PNA did not demonstrate any effect the high dose was able to significantly reduce the mortality rate of the caterpillars (49). Different from these previously conducted studies, our animal experiments were performed by administering PNA into the intestinal tract of chickens. *Campylobacter* is an enteric organism and the chicken model is well established for evaluating *Campylobacter* colonization and emergence of fluoroquinolone resistance (50). To our knowledge, no previous animal studies have been reported, in which PNA is administered orally as a therapeutic or adjunct agent for antibiotics against bacteria.

In contrast to EPIs for which the concentrations required for inhibition of efflux pumps are highly toxic to host cells, the CmeA1 PNA had no apparent adverse effects *in vivo*. The chickens inoculated with the PNA were as healthy as the ones not inoculated with PNA. There were no signs that the PNA caused any physiological effect on the chickens. In addition, PNA alone did not affect *Campylobacter* colonization or development of ciprofloxacin resistant mutants, suggesting that PNA itself doesn't serve as a selection force for *Campylobacter*. On the other hand, the CmeA1PNA showed a potentiating effect on enrofloxacin against two different strains of *C. jejuni* in the chicken model, indicating its potential as a adjunctive therapy for antibiotics. However, more studies are necessary to optimize the treatment regime for PNA. For

example, higher PNA doses may be required to reach an effective concentration in the gut that completely eliminate the development of ciprofloxacin resistant mutants. Additionally, encapsulation of PNA may facilitate effective delivery to the intestinal tract via oral administration. Other routes of administration may also be tested and a time course measurement of PNA in the intestine and other tissues needs to be conducted to better understand the kinetics of PNA in animals. These remain to be determined in future studies.

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CHAPTER 5. GENERAL CONCLUSIONS

The increasing prevalence of fluoroquinolone resistance in *C. jejuni* is a major concern for food safety and public health. Novel strategies are required to combat fluoroquinolone-resistant *Campylobacter*. In this work, we performed both *in vitro* and *in vivo* studies to evaluate the efficacy of the CmeA1 PNA in potentiating fluoroquinolone antibiotic against *C. jejuni*. In the first set of experiments, fluoroquinolone-susceptible *C. jejuni* cultures were subjected to different treatment combinations. The culture treated only with ciprofloxacin developed resistant mutants that rapidly replaced the susceptible population. The ciprofloxacin and PNA co-treated culture, however, did not develop any resistant mutants. This was demonstrated in three independent experiments and was confirmed by spreading the cultures on antibiotic selective plates. Quantitative measurement of growth kinetics further revealed that the PNA not only prevented development of resistant mutants, but also resulted in faster killing of *C. jejuni* by ciprofloxacin. These findings exhibited the efficacy of PNA in preventing the emergence of fluoroquinolone resistant mutants *in vitro*.

In the second set of experiments, the efficacy of PNA in potentiating fluoroquinolone antibiotics was evaluated using an *in vivo* model that involved *Campylobacter* infected chickens. Three chicken trials were conducted in total. In the first trial, 2 different PNA doses, 0.2 μmoles/bird/day and 2 μmoles/bird/day, were evaluated. The result indicated that the low dose did not show a potentiating effect on enrofloxacin, while the high dose reduced the emergence of fluoroquinolone-resistant mutants in the birds treated with enrofloxacin. This finding was further supported by the second trial in which the chickens were infected by a different strain of *C. jejuni*. Again, the group of birds co-treated with ciprofloxacin and PNA showed an initially significantly reduction in the CFU number of fluoroquinolone-resistant mutants. To assess

whether PNA dosing frequencies affect its potentiating efficacy, the third chicken trial was conducted with the dose of 2 µmoles/bird given twice a day. The results from the third trial were similar to the second trial, indicating that 2 doses of PNA/ day did not further improve the efficacy. In all three experiments, chickens treated with ciprofloxacin alone consistently developed fluoroquinoclone-resistant *C. jejuni*, which eventually replaced the susceptible populations in the treated birds. On the contrary, the birds treated by PNA alone were colonized by fluoroquinolone-resistant *Campylobacter* throughout the study, indicating that PNA itself did not influence the development of resistant mutants. The results from these chicken studies demonstrate the potential of the CmeA1 PNA for *in vivo* use as an adjunct therapy. Together, the *in vitro* and *in vivo* data generated in this work reveal that the CmeA1 PNA is able to reduce the emergence of fluoroquinolone resistance in *Campylobacter* and potentiate the efficacy of antibiotic treatment, it can also be further developed for practical use in combating antibiotic resistant *Campylobacter*.

Future Directions

The CmeA1 PNA dosing and treatment regime needs to be optimized further to improve its efficacy *in vivo*. Unlike *in vitro* studies, the conditions in animal intestinal tract are much more complex, which requires additional experiments to evaluate the variables that affect PNA efficacy such as bioavailability. Furthermore, only a single antibiotic dose (50 ppm in drinking water) was used in the chicken trials describe above. Subsequent trials may be conducted using different antibiotic dosages to optimize the antibiotic-PNA combinations that allow for complete elimination of fluoroquiniolone-resistant mutants in treated animals. Additionally, different routes of administration like intra-peritoneal injections may be tested to assess which route provides the best bioavailability of the PNA in the gut. Furthermore, time dependent

measurement of PNA levels in the intestinal tract need to be conducted to evaluate the kinetics of PNA to determine how long it remains in the animal system as that would significantly impact the frequency of the doses. In conclusion, several experiments need to be conducted in the future to determine the most efficacious dose and frequency for PNA co treatment with antibiotics.