Drug-Resistance and Virulence in *Campylobacter jejuni*: Insights from Clinical, Poultry, and Wastewater Interfaces

By

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A thesis submitted to the Department of Mathematics & Natural Sciences in partial fulfillment of the requirements for the degree of

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Declaration

It is hereby declared that the thesis submitted is my/our own original work while completing my degree at BRAC University. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate reference. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution. I have acknowledged all the main sources of help. The correspondence of all the data, outcomes, results presented in the manuscript belong to icddr,b. I am not authorized to publish, share, and present full or any part of this work without prior consent of the correspondence. I would like to request the embargo of my thesis for 24M from the submission date due to an attempt to publish the findings in a reputed journal.

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Approval

The thesis **'Drug-Resistance and Virulence in *Campylobacter jejuni* : Insights from Clinical, Poultry, and Wastewater Interfaces'** submitted by Ayesha Khatun of Fall, 2022 has been accepted as satisfactory in partial fulfilment of the requirement for the degree of Master of science in Biotechnology.

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Ethics Statement

This research was conducted in accordance with ethical guidelines and approved by Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b (International Centre for Diarrhoeal Disease Research, Bangladesh).

Abstract

Campylobacter jejuni is a leading cause of human gastrointestinal infections, primarily transmitted through the consumption of undercooked poultry, the main reservoir of *Campylobacter* species, and via cross-contaminated food and water. The emergence of multidrug-resistant (MDR) *C. jejuni* due to resistance to various antimicrobial agents poses significant global health risks. This research aims to characterize antimicrobial resistance patterns and virulence genes of *C. jejuni* from clinical, poultry and wastewater interfaces in Bangladesh. In this study, fifteen *C. jejuni* isolates were cultured on CHROMagar plates and subjected to phenotypic testing, polymerase chain reaction, and antimicrobial resistance assessment. The Kirby–Bauer disc diffusion method was used to evaluate resistance to nine antibiotic classes, while minimum inhibitory concentrations for ciprofloxacin, erythromycin, and tetracycline were determined by E-test. The isolates showed 100% resistance to ciprofloxacin and erythromycin; 93% to sulfamethoxazole, azithromycin, nalidixic acid, and ampicillin; 87% to ceftriaxone; 73% to cefuroxime; 67% to gentamicin, cefoxitin, and cefixime; 60% to tetracycline; 47% to ampicillin and chloramphenicol; 27% to imipenem; and 7% to amikacin. All isolates were resistant to more than four antibiotic classes, with wastewater isolates showing the highest resistance rates. Resistance to macrolides and fluoroquinolones, which are critical antibiotics for the treatment of Campylobacteriosis, was observed in all isolates. Virulence genes, including *flaA*, *racR*, *cdtA*, *cdtC*, *gyrB*, *parC*, and *wlaN* were investigated using gene-specific PCR. All five wastewater and clinical isolates assessed positive for *flaA* and *parC*. The genes *racR*, *cdtA*, and *cdtC* were present in four clinical isolates, while chicken isolates exhibited *racR* (1/5), *cdtA* (1/5), and *cdtC* (4/5). The *wlaN* gene was detected in one clinical, two chicken, and three wastewater isolates, while the *gyrB* gene was found in one wastewater and two chicken isolates. Overall, virulence gene prevalence was *cdtA* (53%), *flaA* (47%), *wlaN* (40%), *cdtC* (33%), *racR* (33%), *parC* (33%), and *gyrB* (20%). In Bangladesh, widespread self-medication with antibiotics and treatment failures due to resistance exacerbate the problem. However, MDR *C. jejuni* and their resistance patterns at the clinical-poultry-wastewater interface remain poorly studied. This study provided valuable insights into the antimicrobial resistance profiles and virulence determinants of MDR *C. jejuni*, a significant contributor to diarrheal mortality in Bangladesh, thereby enhancing understanding of its pathogenesis and implications for public health.

Keywords: Multi-drug resistant, *Campylobacter jejuni*; gastroenteritis; antibiotic susceptibility test; minimum inhibitory concentration; resistance genes; virulence genes

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Dedication

I dedicate this work to my beloved husband.

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Abbreviation

GBS	Guillain-barré syndrome
PCR	Polymerase chain reaction
MFS	Miller fisher syndrome
BHI	Brain heart infusion
TE Buffer	Tris-EDTA buffer
DW	Deionized water
dNTP	Deoxynucleotide triphosphate
bp	Base pair
et al	And others
DD	Disk Diffusion
MDR	Multi-Drug Resistant
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra acetic acid
E-Test	Epsilometer
g	Gram
Kb	Kilobase
L	Liter
Fig.	Figure
ml	Milliliter
Min.	Minute
O/N	Over night
CJ	*Campylobacter jejuni*
Icddr,b	International centre for diarrheal disease research, Bangladesh
CLSI	Clinical and laboratory standards institute
VFDB	Virulence Factor Database

Chapter 1

Introduction

Campylobacter is one of the leading foodborne pathogens which causes human bacterial gastroenteritis(Platts-Mills & Kosek, 2014). *Campylobacter* species can cause mild to severe diarrhea, with loose, watery stools often followed by bloody diarrhea. *Campylobacter* infections are mostly acquired from the consumption of contaminated raw or undercooked chicken, unpasteurized milk, or untreated water (Pitkänen, 2013; Skarp et al., 2016). Although poultry is a known reservoir for *Campylobacter spp.*, water has been shown to play an important role in the transmission of *Campylobacter* illnesses, either directly or indirectly by infecting animals (Sales-Ortells & Medema, 2015). Waterborne *Campylobacter* outbreaks have been reported in numerous countries (Kuhn et al., 2017). Over 400 million cases of Campylobacteriosis are reported globally each year (Kaakoush et al., 2015a). In Europe, up to 246,307 persons were infected with Campylobacteriosis in 2016, while an estimated one million people are sick in the United States each year (Chlebicz & Śliżewska, 2018). *Campylobacter* infections are frequent in Asia, the Middle East, and Africa, especially in children (Johnson et al., 2017; Sainato et al., 2017). *Campylobacter* is a highly pathogenic bacteria that can cause urinary tract infections, septicemia, and a variety of neuropathies such as reactive arthritis, Guillain-Barre syndrome (GBS), irritable bowel syndrome, and Miller-Fisher syndrome (MFS) and abortion in pregnant women (Heikema et al., 2015; Kaakoush et al., 2015a; Karikari et al., 2017; Nyati & Nyati, 2013). It is estimated that *Campylobacter spp.* is responsible for 400-500 million cases of diarrhea each year, worldwide (Luangtongkum et al., 2009). The incidence and prevalence of Campylobacteriosis have increased in both developed and developing countries over the last 10 years. In the 2010 Global Burden of Disease Study estimated that 7.5 million disability-adjusted life years with Campylobacteriosis, which was associated with more than Shigella (7.1 million) and enterotoxigenic *E. coli* (6.9 million) (Platts-Mills & Kosek, 2014). In the United States, the annual number of Campylobacteriosis cases, based on 10 years of outbreak data (1998 to 2008), was estimated to be 845,024 cases, resulting in 8,463 hospitalizations and 76 deaths (Batz et al., 2012) and current evaluation of the epidemiology of Campylobacteriosis in 27 European Union (EU) state members indicated the incidences of *Campylobacter* infections to range from 29.9 to 13,500

per 100,000 population in 2009 (Platts-Millsa et al., 2014). In a different study, *Campylobacter jejuni* was reported to be significantly associated with moderate to severe diarrhea in children from Kolkata, India, Mirzapur, Bangladesh, and Karachi, Pakistan (Kotloff et al., 2013). A rise in *Campylobacter* infections has been observed in both developed and developing nations (Kaakoush et al., 2015b). *C. jejuni* is the most common species of *Campylobacter* and is commonly associated with diarrhea or other bacterial illnesses (Wieczorek & Osek, 2013). Other species involved in gastroenteritis include *C. coli, C. lari,* and *C. upsaliensis* (Kaakoush et al., 2015b).

In Bangladesh, most of the studies were limited to isolating *Campylobacter* species from patients with diarrhea. However sufficient data is not available yet on the prevalence of *C. jejuni* and multi-drug resistance pattern and virulence genes of *C. jejuni* in multiple sources. Also, poultry and layer chicken in Bangladesh is harvested and consumed widely for meat and egg. The growing antibiotic resistance of *Campylobacter* isolates in humans, animals, and the environment is rapidly becoming a major public health issue (Ghosh et al., 2013; Ghunaim et al., 2015; Shobo et al., 2016). Antimicrobial treatment is limited to severe cases, the upward trend of multi-drug resistance (MDR) is a concern. However, no multi-source MDR study on *Campylobacter* in Bangladesh was reported. Therefore, this study aimed to investigate AMR patterns and virulence genes prevalence in clinical-livestock-environmental interfaces in Bangladesh. In this study, we looked out AMR profile of nine different classes of drugs. The recommended antimicrobials for treating *Campylobacter* infections are macrolides, fluoroquinolones, and tetracyclines (Pérez-Boto et al., 2014). However, resistance to these empirical medicines has been recorded in several countries (Garcia-Migura et al., 2014; Ghosh et al., 2013; Pérez-Boto et al., 2014; Redgrave et al., 2014; Shobo et al., 2016). Tetracycline resistance is typically related to the *tetO* gene, whereas fluoroquinolone resistance is caused by mutations in the *gryA* or *parC* genes (Laprade et al., 2016; Pérez-Boto et al., 2014). Resistance to macrolides is commonly caused by mutations at locations 2074 or 2075 of domain V in the *rrn* gene, which encodes the 23S rRNA.

This study, therefore, evaluated the antibiotic susceptibility profile and virulence genes identification of MDR *C. jejuni* isolates from clinical, livestock, and wastewater isolates obtained in Dhaka, Bangladesh. It promotes a One Health approach to control AMR spread by investigating causes. Establishing such a link could aid in identifying places of intervention for the prevention of *Campylobacter*-related illnesses, especially in resource-limited settings. These findings help to shape public health policies, promote antibiotic stewardship, and raise

awareness about responsible antibiotic use and food safety. This study establishes a baseline for future monitoring and promotes coordinated initiatives thereby improving healthcare outcomes to minimize AMR prevalence in Bangladesh.

1.1. Historical Background of *Campylobacter jejuni*

Campylobacter is named after from Greek ancient "curved rod" that resembles the shape of the organisms (Henry, 2013). The earliest report on *Campylobacter* is attributed to Theodore Escherich, who found and reported non-culturable spiral-shaped bacteria in 1886 (Debruyne et al., 2008). McFadyean and Stockman discovered these germs in deformed cow fetuses in 1913. The bacteria that Smith and Orcutt identified in 1927 from the feces of diarrheal calves gave rise to the term Vibrio jejuni. Seventeen years later, in 1944, Doyle discovered a distinct vibrio from the excrement of diarrheal pigs, and Vibrio coli was classified (Vandamme et al., 2010). In 1906, two British veterinarians found "vast quantities of a peculiar species" in a pregnant sheep's vaginal mucus for the first time (Zilbauer et al., 2008). After surpassing Salmonella a few years ago, *Campylobacter* has been named the leading cause of bacterial foodborne infections in affluent nations, with a heavy economic burden (EFSA and ECDC, 2016). There have been substantial changes made to the taxonomic structure of the genus *Campylobacter*, and there are still some questions regarding the current taxonomy of the genus that need to be answered (Henry, 2013).

1.2. Taxonomy

Taxonomy (from Ancient Greek *taxis*), meaning 'arrangement', and (nomia), meaning 'method') is the science of defining and naming groups of biological organisms based on shared characteristics. Organisms are grouped into taxa (singular: taxon) and these groups are given a taxonomic rank; groups of a given rank can be aggregated to form a super-group of higher rank, thus creating a taxonomic hierarchy. The principal ranks in modern use are a domain, kingdom, phylum (division is sometimes used in botany in place of phylum), class, order, family, genus, and species. The Swedish botanist Carl Linnaeus is regarded as the father of taxonomy, as he developed a system known as Linnaean taxonomy for the categorization of organisms and binomial nomenclature for naming organisms. The family *Campylobacteraceae* (proposed in 1991) includes four closely related genera; *Campylobacter*, *Arcobacter*, *Dehalospirillum*, and

Sulfurospirillum. The genus *Campylobacter* currently contains 26 species of which 19 have been isolated from humans. There are also 10 subspecies of which nine are from humans (Parte, 2014). However, the two most remarkable species that are responsible for over 95% of all human *Campylobacter* infections worldwide are *C. jejuni* and *C. coli* (Wilson et al., 2009). The taxonomy of *Campylobacter* is:

Table 1.1. Taxonomy of *Campylobacter jejuni*

Domain:	**Bacteria**
Phylum:	**Proteobacteria**
Class:	**Epsilon proteobacteria**
Order:	**Campylobacterales**
Family:	***Campylobacteraceae***
Genus:	***Campylobacter***
Species:	***Campylobacter jejuni***

1.3. Phenotypic Characteristics of *Campylobacter jejuni*

Campylobacter jejuni is a non-sporing gram-negative microaerobic bacterium from the *Campylobacteraceae* family (Henry, 2013). They move in a corkscrew-like pattern, producing motile, spiral-shaped rods ranging in width from 0.2 to 0.9 meters and length from 0.5 to 5 meters (Figure 1.1). The presence of one unsheathed polar flagellum at the cell's end gives the bacterium a thin "S" shape, its most distinguishing feature. Anaerobic growth conditions, optimal growth at 42°C, and inability to grow below 30°C are all unique to *C. jejuni* and relate to the absence of the cold shock protein gene, which is involved in many bacteria's low-temperature adaptation (Hazeleger et al., 1995). Other prominent phenotypic traits of *C. jejuni* include nitrate reduction to

Figure 1.1. Getty images showing colored scanning electron micrograph (SEM) of *C. jejuni*

nitrite, hippurate hydrolysis, the absence of urease, nalidixic acid sensitivity, cephalothin resistance, and the inability to metabolize carbohydrates (Levin, 2007)

1.4. Genomic Structure

Several numbers of genomes *Campylobacter* species have been sequenced, beginning with *Campylobacter jejuni* in 2000 (Fouts et al., 2005; Parkhill et al., 2000). These genome studies have identified molecular markers specific to members of *Campylobacter*. Additionally, several markers were found in all *Campylobacter* species except for *C. fetus*, the most distantly related species. Many markers were also found that were conserved only between *C. jejuni* and *C. coli*, indicating a close relationship between these two species (Grant et al., 1993; Gupta, 2006).

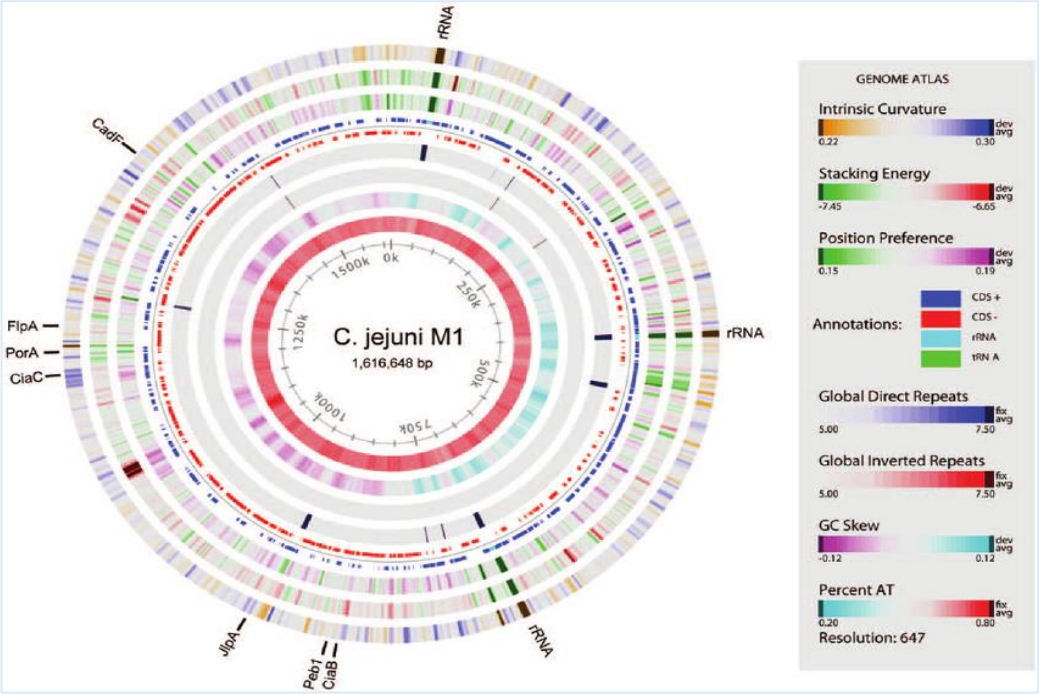

Figure 1.2. Genome Atlas of *C. jejuni* M1 (Friis et al., 2010)

Similar studies have investigated the genes responsible for motility in *Campylobacter* species. All *Campylobacter* species contain two flagellin genes in tandem for motility *flaA,* and *flaB*. These genes undergo intergenic recombination, further contributing to their virulence (Grant et al., 1993). There are a growing number of quinolone-resistant strains. Evidence suggests this is caused by an overuse of this class of antibiotics in animal agriculture (Crushell et al., 2004).

1.5. Epidemiology

Campylobacter jejuni is one of the most well-known pathogenic bacteria in clinical trials of acute gastroenteritis, where fecal cultures were acquired. Indeed, the number of cases much exceeds the overall number of diarrheal diseases aggravated by *Salmonella, Shigella,* and *Escherichia coli* 0157:H7 (Blaser et al., 1983). Despite their significance, cultures of this bacterium are rarely frequently evaluated in many hospital laboratories. Furthermore, because *C. jejuni* production is more complicated and expensive than cultivating other *Shigella* and *Salmonella* species, infection with *C. jejuni* is anticipated to be greatly reduced (Allos & Blaser, 1995). After rotavirus (20%), *C. jejuni* has been identified as the second most common entero-pathogen in diarrheal patients of young children in Bangladesh (17%). In Bangladesh, a case-control study carried out at icddr, b revealed that *C. jejuni* was the second most common Enteropathogens with the isolation rate of 17.4% from all age groups. Whereas in the US, 11% (2 million) of the population acquires Campylobacteriosis each year and in an industrialized country, *C. jejuni* is isolated from 5% to 16% of children under 5 years. Thus, it appears to be one of the major pathogens associated with diarrhea in Bangladesh (Schnee et al., 2018). *Campylobacter*-related gastroenteritis incidence rates vary significantly across different rich countries (Amato-Gauci & Ammon, 2007). Young children had the highest rate of symptomatic infections, followed by young adults. Furthermore, there is a strong seasonality, with a summer peak between June and September (Olson et al., 2008). According to CDC estimates (Tauxe et al., 1988), the annual prevalence of *C. jejuni* infection is estimated to be 5-6 per 100,000 persons. However, according to more thorough data from population-based studies, the annual frequency is 1000 per 100,000 people (Allos & Blaser, 1995). Similarly, the CDC surveillance system detected only one case in an outbreak with an estimated 1000 patients (Sacks et al., 1986). Based on these data, the number of symptomatic *C. jejuni* infections in the United States is predicted to be -2.5 million each year. Most of the *Campylobacter* cases are sporadic and not related to outbreaks. The most prevalent source of *Campylobacter* infection is interaction with agricultural animals such as cattle, poultry, and pigs (38). Untreated water, raw milk, and milk products can all cause infections (Olson et al., 2008).

1.6. Reservoirs, route of Transmission, Environmental Distribution of *Campylobacter*

Campylobacter infection is considered hyperendemic in developing nations, with environmental and food contamination being the primary causes of infection (Coker et al., 2002). In contrast, food-producing animals are thought to be the principal cause of *Campylobacter* infections in people in affluent countries such as the United States. *Campylobacter* illness can be transmitted through cross-contamination anywhere from animal farms to commercial food processing. The most common modes of transmission include fecal-oral, ingestion of contaminated food or water, and eating raw meat. Additionally, intake of poultry, beef, and pork products is the primary cause of human foodborne illness. Foods associated with Campylobacteriosis include raw or undercooked poultry, raw dairy products, and infected produce (Scallan et al., 2011). *Campylobacter* is sensitive to the stomach's natural production of hydrochloric acid; hence, the infectious dosage is quite large, and the germs rarely cause sickness when a person is exposed to less than 10,000 organisms (Scallan et al., 2011). In Europe, retail chicken meat is infected with *Campylobacter jejuni* (Bull et al., 2006).

Nonetheless, those who take antacids (for example, those with gastritis or stomach ulcers) are more likely to get the disease from a smaller number of organisms since these medications suppress natural gastric acid. Poultry is thought to account for 50-70% of human *Campylobacter* infections. Poultry includes broilers, laying hens, turkeys, ducks, and ostriches (Blaser & Engberg, 2008). *C. jejuni* primarily colonizes in chicken and lives in the colon, although it can also be found in crops. *Campylobacter* transmission is common during pre-harvest circumstances. Colonization in broiler chicks has been shown to increase the chance of horizontal transmission of *C. jejuni* infection. Broiler chick colonization is expected to occur no sooner than seven days of age (El-Shibiny et al., 2005; Moore et al., 2005). Although young animals are very sensitive to *C. jejuni* colonization, older broiler chicks approaching processing age have a higher percentage of *Campylobacter* colonization. Although horizontal transmission of the flock occurs quickly, colonization of the flock can take many weeks (Horrocks et al., 2009). Unpasteurized bovine milk and milk products are typical vectors of *Campylobacter* foodborne illness transmission. In a study of cattle on a dairy farm, 12% of raw milk samples were found to be infected with *C. jejuni*, perhaps due to contact with bovine feces, polluted water, or direct contamination because of mastitis (Epps et al., 2013).

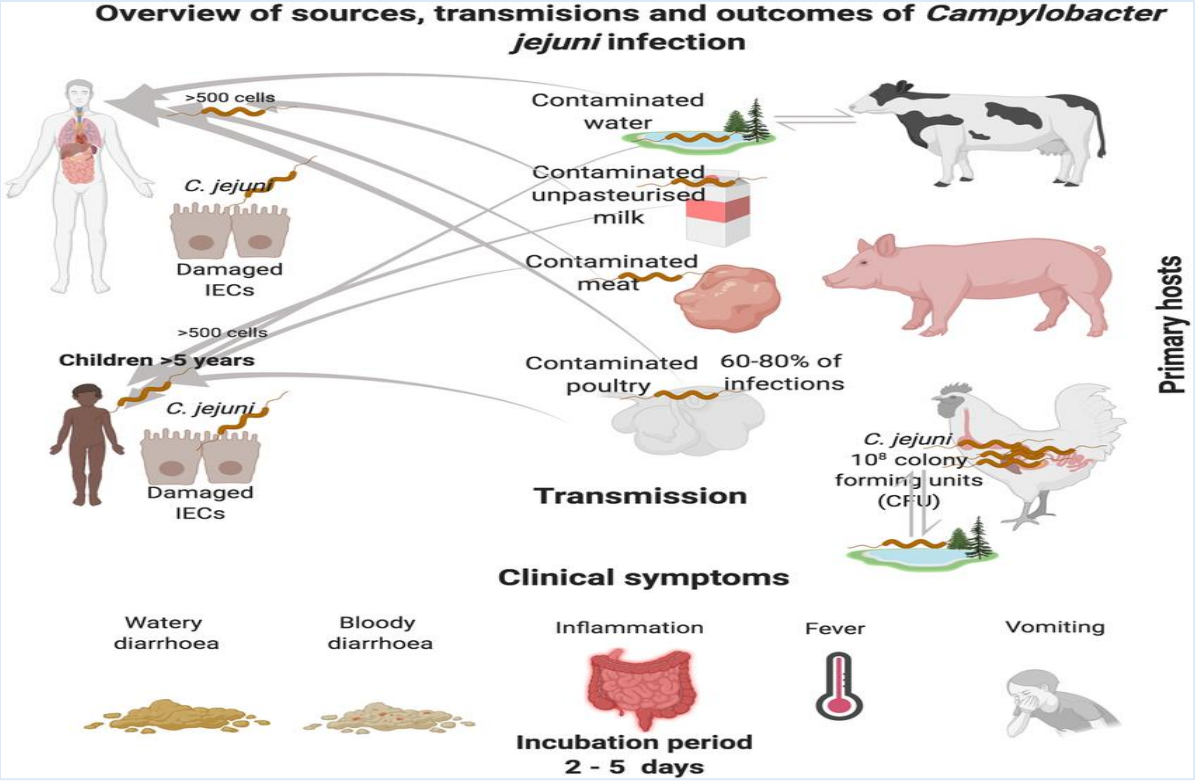

Figure 1.3. Overview of sources, transmission, and outcomes of *Campylobacter jejuni* infection (Elmi et al., 2021).

Campylobacter incidence is found to be lower in forage-fed cattle (mainly dairy cattle) than in feedlot cattle. The following factors may contribute to the high occurrence of *Campylobacter* in feedlot cattle: (1) increasing stocking densities, (2) continual contact with other animals' excrement, and (3) frequent shared access to communal feeding and water troughs (Beach et al., 2002; Horrocks et al., 2009). These studies discovered a higher prevalence of *Campylobacter* in feedlot cattle (68%) than in adult cattle from pasture (7.3%), and others discovered that prevalence rates in fed cattle increased from 1.6% near or upon entry to the feedlot to 63% near the end of the finishing period (Blaser et al., 1980). The prevalence remained very consistent whether tests were performed before or after shipment to processing (Epps et al., 2013). The number of pigs colonized by *Campylobacter* is closer to that of cattle than poultry. *Campylobacter coli*, which generally lives in pig intestines, can also be discovered on pork products. As with cattle and poultry, environmental circumstances play a crucial role in *Campylobacter* transmission in pigs. There is an elevated risk of *Campylobacter* infection and illness transmission in finishing units and breeding farms (Young et al., 2000). *Campylobacter* colonization and infection of pigs grown in organic outdoor production systems vary significantly.

1.7. Pathogenesis

Campylobacter infections can cause mild to severe diarrhea, with stool containing blood and leukocytes. Additional symptoms may include fever, nausea, and stomach pain. *Campylobacter* enteritis has an incubation period of two to seven days and is usually self-limiting. Patients with severe instances are treated with erythromycin. Black et al. (1988) found that a low dose of *Campylobacter jejuni* (500-800) is enough to cause diarrhea, with greater doses increasing the frequency of cases (Black et al., 1988). *C. jejuni* infections can cause Guillain-Barré syndrome, an acute demyelinating polyneuropathy. GBS develops from gastrointestinal illness and is marked by flaccid paralysis. O-side chain serotyping investigations have demonstrated a relationship between specific *Campylobacter* serotypes and GBS. The majority of GBS cases in the United States result from serotype O:19 infections. The exact sequential steps of *C. jejuni* infection to *C. jejuni-mediated* enteritis are unknown. What is known are the requirements for *C. jejuni* virulence: *C. jejuni* infections are commonly acquired by handling and consuming undercooked chicken and drinking unpasteurized milk and polluted water. Human illness with *C. jejuni* ranges from mild to severe diarrheal disease, the latter of which is characterized by the presence of blood and leukocytes in stool specimens (Konkel et al., 2001).

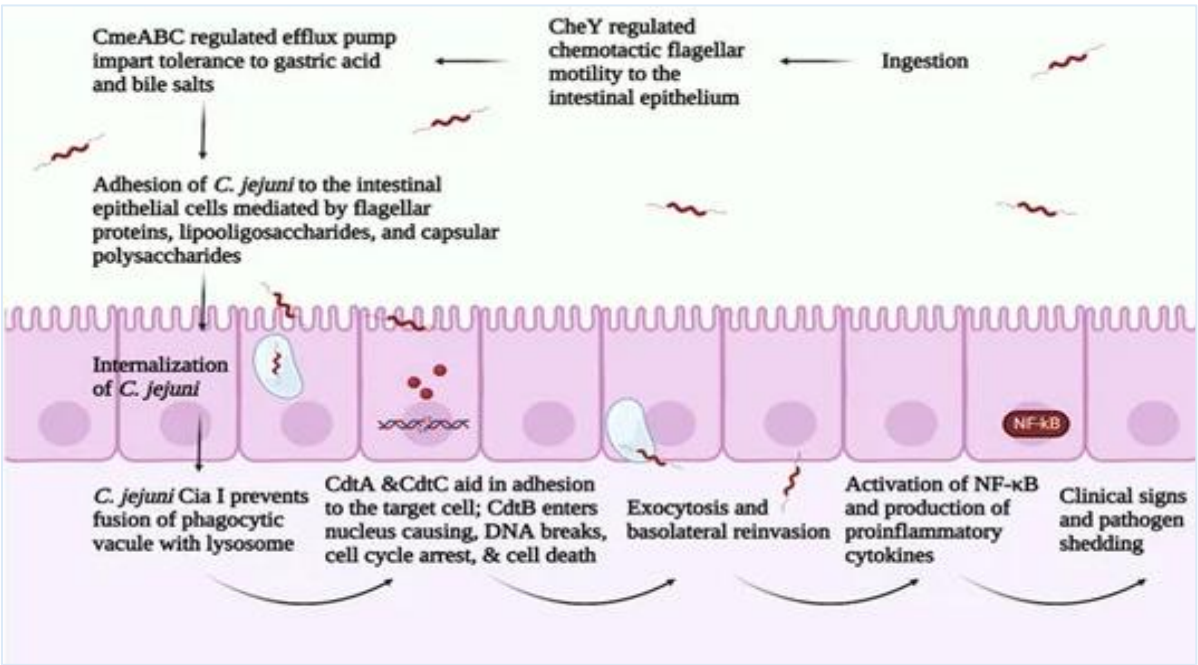

Figure 1.4. Overview of *Campylobacter jejuni* pathogenesis (Al Hakeem et al., 2022)

1.8. Campylobacteriosis and its treatment

Campylobacteriosis is an infection caused by *Campylobacter* species, predominantly *Campylobacter jejuni* and, to a lesser extent, *Campylobacter coli*. It is a leading cause of bacterial gastroenteritis worldwide and is typically transmitted via contaminated food, water, or direct contact with animals (Allos & Blaser, 1995). *C. jejuni* infection usually causes an acute, self-limited gastrointestinal disease with diarrhea, fever, and abdominal cramps. *Campylobacter* infection is clinically indistinguishable from acute gastrointestinal infections caused by other bacterial pathogens, including *Salmonella*, *Shigella*, and *Yersinia*. Most patient's diarrhea is either loose and watery or severely bloody, with 8-10 stool movements per day at the height of illness (Blaser et al., 1983). In other patients, diarrhea is mild, and stomach cramps and pain are the major symptoms; this can lead to an incorrect diagnosis of acute abdomen and unnecessary laparotomy. More than 90% of patients report fever, which can be low-grade or higher than 40°C and last for up to a week. Even without special antibiotic treatment, the sickness normally resolves by that point. However, individuals may experience a prolonged, recurrent diarrheal sickness that lasts many weeks (Kapperud et al., 1992). Although *Campylobacter* is infrequently seen in the stools of healthy people, depending on the population studied, up to half of those infected during outbreaks are asymptomatic (Calva et al., 1988). Mild to moderate cases are treated with oral rehydration salts (ORS) and fluids to prevent dehydration. Loperamide and other anti-motility medications should be used with caution when treating non-bloody diarrhea. Serious cases or high-risk individuals may require antibiotics such as macrolides, fluoroquinolones or tetracyclines (Tikhomirova et al., 2024), usually for 3-7 days, with susceptibility testing recommended due to rising resistance. Complications such as reactive arthritis are treated with NSAIDs, whereas Guillain-Barré Syndrome (GBS) may require plasmapheresis or intravenous immunoglobulin. Proper sanitation, food safety, and avoiding unpasteurized milk are all crucial for infection prevention.

1.9. Introductory points on antibiotics and their resistance

1.9.1. Antibiotics and their mode of actions

An antibiotic is a low molecular weight substance produced by microorganisms and toxic in low concentration to other susceptible microorganisms. Production of antibiotics seems to be restricted to two classes of microorganisms, bacteria, and fungi. Antibiotics can be natural,

semi-synthetic or synthetic. There are several classes of antibiotics such as ß-lactams, aminoglycosides, peptides, acetogenin, macrolides etc. The mode of action of antibiotics may be bacteriostatic or bactericidal. The type of antibiotics we will take depends on the type of infection we have and what kind of antibiotics are to be effective. The main classes of antibiotics are Aminoglycosides, Cephalosporins, Fluoroquinolones, Macrolides, Penicillin, Tetracycline, Carbapenems, Sulfonamides etc.

1.9.2. Cephalosporins

Cephalosporin resistance in *Campylobacter jejuni* is a growing problem, particularly as this pathogen is found in both environmental and clinical settings in Bangladesh. Resistance is generally caused by beta-lactamase enzymes (e.g., blaOXA genes), which hydrolyze the beta-lactam ring, leaving cephalosporins ineffective. Another important mechanism is the cmeABC efflux pump, which actively expels cephalosporins, lowering drug concentrations within bacterial cells (Griggs et al., 2009). Mutations in penicillin-binding proteins (PBPs) can also decrease the affinity for cephalosporins, reducing their efficiency.

1.9.3. Macrolides: Erythromycin, Azithromycin

Macrolide resistance in *Campylobacter* is the result of modification of the ribosome target binding site by mutation of the 23S rRNA or changes in resulting proteins at the site rather than target methylation or enzymatic drug modification seen in other bacterial species. Base substitutions at positions 2074 and 2075 of the adenine residues in all three copies of the 23S rRNA gene (rrnB operon) in Campylobacter are the most common mutations conveying erythromycin resistance. The A2074C, A2074G, and A2075G mutations are found to confer a high-level resistance to macrolide antibiotics (erythromycin MIC > 128 mg/L) in *C. jejuni* and *C. coli*. Resistance to erythromycin tends to correspond with cross-resistance to other macrolides (e.g., azithromycin and clarithromycin). Resistance to macrolides among *Campylobacter* isolates may also be caused by modifications of the ribosomal proteins L4 and L22. Several modifications have been reported, and it is possible that they might be associated with low-level resistance to the macrolides. However, the exact role of these L4 and L22 modifications (mutations, insertions, and deletions) is still not clear (Avrain et al., 2004; Cagliero et al., 2006; Caldwell et al., 2008; Corcoran et al., 2006). Efflux is another common mechanism causing macrolide resistance in *Campylobacter* bacteria where at least eight different efflux systems have been identified. One of them is CmeABC multidrug efflux pump

that interacts constructively with specific mutations, even in the absence of any other factor affecting resistance (Cagliero et al., 2006).

1.9.4. Tetracyclines

Resistance to tetracyclines in *Campylobacter* is conferred by the *tet(O)* gene, which is widely present in both *C. jejuni* and *C. coli* (Black et al., 1988; Friedman et al., 2004; Revez et al., 2014). Except the $tet(O)$ marker, no other *tet* resistance genes have been found in *Campylobacter*. Tetracycline binds to Mg2+ cations to pass through outer membrane porins and then, in the periplasmic space, dissociates from magnesium and moves passively into the cytoplasm to bind to discrete sites on the ribosomal 30Ssubunit (Chopra & Roberts, 2001). Its primary antimicrobial effect takes place by direct steric hindrance by binding to the A site in the 30Ssubunit, thus hindering the movement of transfer RNA and inhibits peptide elongation (Harms et al., 2003). The *tet(O)* gene, which encodes ribosomal protection proteins (RPPs), is located on a self-transmissible plasmid of a molecular size from 45 to 58 kb. The *tet(O)* gene has been shown to confer extremely high levels of tetracycline resistance (512 mg/L) (Gibreel et al., 2004). Recent study demonstrates that this protein recognizes an open A site on the bacterial ribosome and binds it in such a manner that it induces a conformational change that results in the release of the bound tetracycline molecule (Connell et al., 2003). Furthermore, the conformational change persists for an extended period, thus allowing for continued protein elongation in an efficient manner (Connell et al., 2003). Tetracyclines, which are the subject of RPP-mediated resistance, including *tet(O)*, bind to the ribosome and inhibit accommodation of the aminoacyl tRNA (aa-tRNA) into the ribosomal A site and, therefore, prevent the elongation phase of protein synthesis (Chopra, 1985).

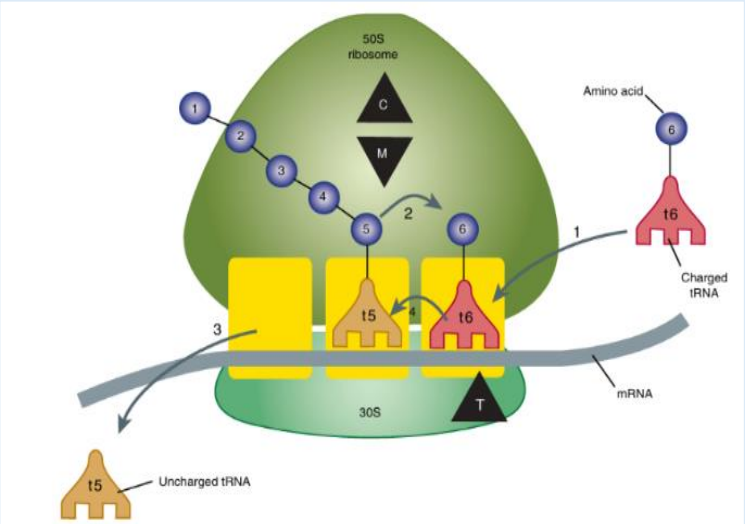

Figure 1.5. Steps in bacterial protein synthesis and targets of several antibiotics (Chloramphenicol, Macrolides and Tetracycline) (Vanderah, 2024).

1.9.5. Aminoglycosides: Amikacin, Gentamicin

Resistance to aminoglycosides in *C. jejuni* is becoming a significant concern. This resistance arises from the acquisition of specific resistance genes and mutations that interfere with aminoglycoside efficacy. The main mechanism involves aminoglycoside-modifying enzymes (AMEs), particularly phosphotransferases (APH) and adenyltransferases (ANT). These enzymes chemically modify aminoglycosides, reducing their binding affinity to bacterial ribosomes, thus preventing their action (Wieczorek & Osek, 2013). Additionally, mutations in the ribosomal binding sites of aminoglycosides further contribute to resistance. Another mechanism involves efflux pumps, such as cmeABC, which expel aminoglycosides from bacterial cells, reducing intracellular drug concentrations.

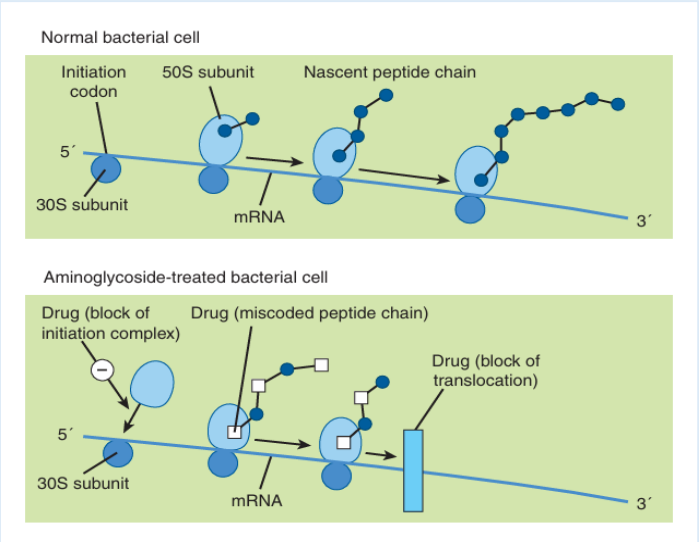

Figure 1.6. Putative mechanisms of action of the aminoglycosides in bacteria (Vanderah, 2024)

1.9.6. Penicillins: Ampicillin, Amoxicillin

Due to the widespread use of beta-lactam antibiotics, Penicillin resistance is a growing concern. Penicillins, like other beta-lactams, target bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), which are essential for cell wall stability. However, *C. jejuni* has developed several mechanisms to evade the effects of Penicillins, primarily

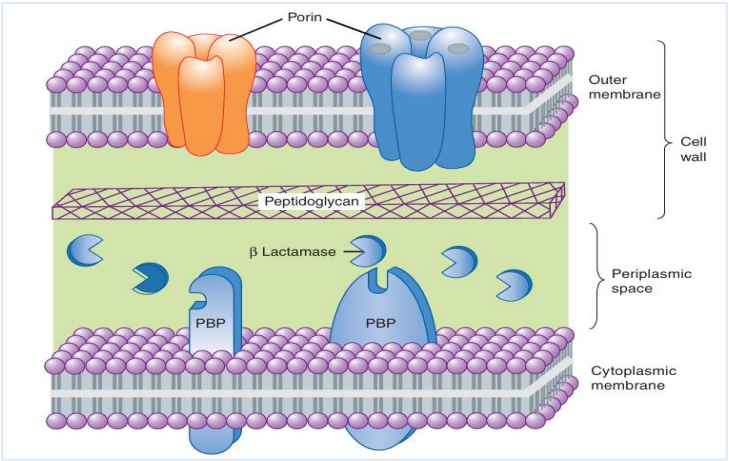

Figure 1.7. Mechanism of action of β-lactam antibiotics (Vanderah, 2024)

through resistance genes and adaptive mutations (Wieczorek & Osek, 2013). The most common mechanism involves beta-lactamase enzymes, such as those encoded by the *blaOXA* genes, which hydrolyze the beta-lactam ring in penicillin, rendering it ineffective. These enzymes can break down penicillin and related antibiotics before they reach their bacterial targets (Griggs et al., 2009). Additionally, mutations in penicillin-binding proteins (PBPs) can alter their structure, reducing drug binding affinity and thus lowering susceptibility to Penicillins.

1.9.7. Quinolones: Nalidixic Acid, Ciprofloxacin

The primary mechanism of quinolone resistance in *C. jejuni* involves mutations in the *gyrA* gene, which encodes subunit A of DNA gyrase, a crucial enzyme for DNA replication (Wieczorek & Osek, 2013). Mutations in *gyrA*, particularly at the Thr-86 position, reduce the binding affinity of quinolones, rendering them ineffective (Luangtongkum et al., 2009). Additionally, efflux pumps, such as the *cmeABC* system, play a role in reducing intracellular concentrations of quinolones by actively expelling them from the bacterial cell, thereby contributing to resistance. The combined effects of *gyrA* mutations and efflux pump activity make quinolone resistance highly prevalent in *C. jejuni* isolates, especially in areas with high antibiotic use (Gupta, 2006).

1.9.8. Carbapenem: Imipenem

Carbapenem resistance in *C. jejuni* primarily involves beta-lactamase enzymes encoded by genes like *blaOXA-61*. These enzymes hydrolyze the beta-lactam ring in carbapenems, preventing them from effectively inhibiting bacterial cell wall synthesis. Additionally, efflux pumps (e.g., *cmeABC*) can contribute to reduced carbapenem susceptibility by decreasing drug accumulation within bacterial cells (Luangtongkum et al., 2009)

1.9.9. Sulfonamide: Sulfamethoxazole

Sulfonamides act as competitive inhibitors of dihydropteroate synthase (DHPS), an enzyme involved in folate synthesis, which is crucial for bacterial growth and replication. However, *C. jejuni* has developed resistance to sulfonamides primarily through the acquisition of specific sulfonamide-resistant genes, notably *sul1* and *sul2*. The *sul1* and *sul2* genes encode modified versions of the DHPS enzyme that have a reduced affinity for sulfonamides, allowing bacterial cells to bypass the inhibitory effects of the drugs (Sunde & Norström, 2005). These genes are

often located on mobile genetic elements such as plasmids, facilitating horizontal gene transfer among bacterial populations and contributing to the spread of resistance.

1.9.10. Chloramphenicol

Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, thus interfering with peptide bond formation. However, resistance to chloramphenicol in *C. jejuni* primarily occurs through the acquisition of chloramphenicol acetyltransferase (*CAT*) genes, such as cat (Roberts & Schwarz, 2017). The *cat* gene encodes an enzyme that inactivates chloramphenicol by acetylation, preventing it from binding to the ribosome. Additionally, efflux pumps, like the *cmeABC* system, contribute to resistance by expelling chloramphenicol from bacterial cells, reducing its intracellular concentration (Luangtongkum et al., 2009)

1.10. Bacterial resistance to antibiotics

Antibiotic resistance is the ability of bacteria or other organisms to withstand the effects of antibiotics. Antibiotic resistance occurs when bacteria adapt in a way that diminishes or eliminates the effectiveness of medications, chemicals, or other agents used to treat or prevent infection. The germs live and continue to reproduce, inflicting additional harm. Antimicrobial resistance encompasses resistance to medications used to treat illnesses caused by other bacteria such as parasites (e.g., malaria), viruses (e.g., HIV), and fungi (e.g., *Candida*). In 1969, the US surgeon general described this enthusiasm in the following momentous words to Congress: "The time has come to close the book on infectious disease." While many praised this concept, the realities of infectious diseases would take an unanticipated and radically different turn in the coming period. Even before penicillin was utilized clinically, Abraham and colleagues found an enzyme capable of degrading it. By 1950, half of all S. aureus isolates were resistant to penicillin. Penicillin resistance emerged first in hospital-acquired Staphylococcus, but by the late 1960s it had spread to community-acquired infections as well.

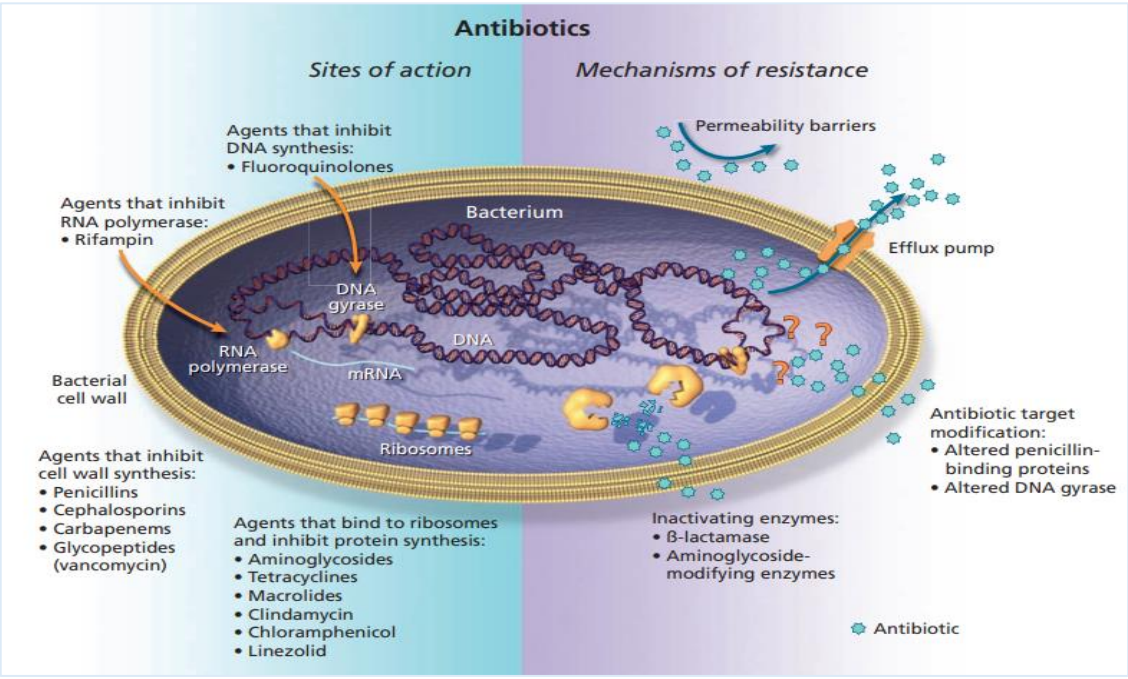

Figure 1.8. Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents (Neu, 1992).

1.11. Mechanism of Antibiotic Resistance

Antibiotic resistance (AMR) is the ability of bacteria to resist the effects of past antibiotic treatments. Resistance to penicillins and other β-lactams is due to one of four general mechanisms: (1) inactivation of antibiotic by β-lactamase, (2) modification of target PBPs, (3) impaired penetration of drug to target PBPs, and (4) efflux. Beta-lactamase production is the most common mechanism of resistance. The phrase more particularly refers to bacteria that become resistant to antimicrobial agents. Resistance arises through decreased cell wall permeability, such as the loss of outer membrane D2 porin in imipenem-resistant *Pseudomonas aeruginosa*. Enzymatic inactivation occurs via β-lactamases that deactivate penicillins, observed in *Staphylococcus aureus*, *Haemophilus influenzae*, and *Escherichia coli*. Changes in targets, like reduced affinity of penicillin-binding proteins, are evident in *Streptococcus pneumoniae*. Increased efflux pump activity leads to the expulsion of antibiotics, such as tetracycline or fluoroquinolones, in *Staphylococcus aureus*. Lastly, bacteria may bypass antibiotic inhibition by utilizing environmental products like thymidine, bypassing internal synthesis pathways. These mechanisms collectively enable bacterial survival against antimicrobial agents.

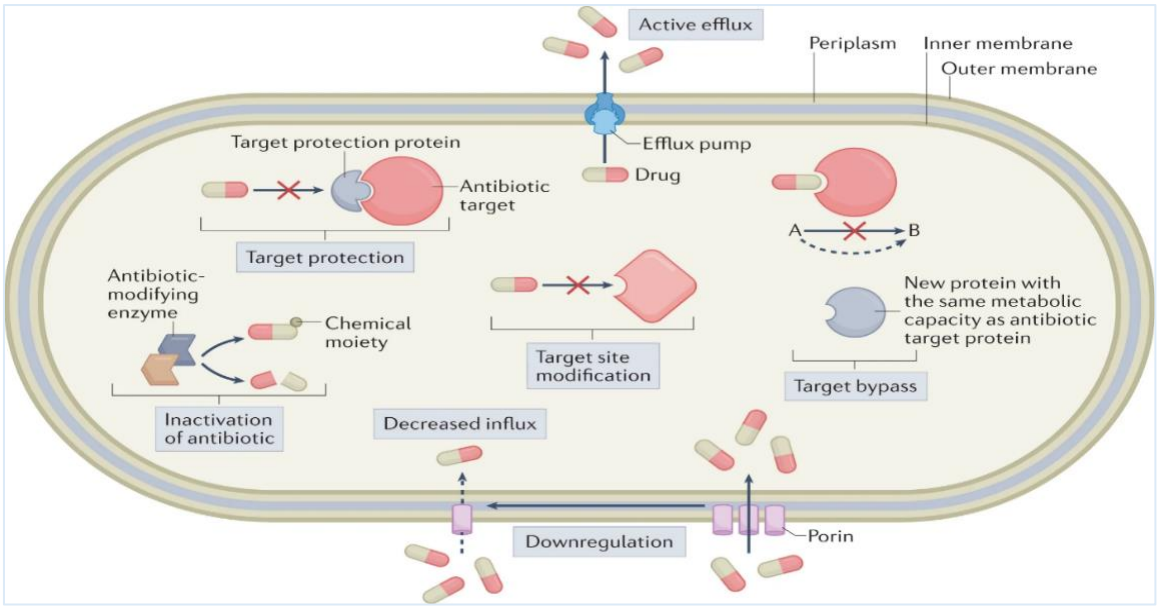

Figure 1.9. Molecular mechanisms of antibiotic resistance (Darby et al., 2023)

1.12. Emergence of Antibiotic Resistance

The expression of antibiotic resistance to antibiotics or antimicrobial agents is influenced by several factors, including the bacterial strain's level of resistance expression and its ability to live via resistance mechanisms (Hwang et al., 2014; Orfali et al., 2022). Microbial strains may have intrinsic resistance or react aggressively to transgene expression from one bacteria to another via plasmids, transgenes, genetic elements, and phages, or to changes in cell genes (chromatin instability) that cause cross-resistance (Davies & Davies, 2010). Biochemical mechanisms work behind the scenes to protect the bacterial cell wall against diverse chemicals, resulting in target modification, enzymatic degradation, and reduced or increased efflux pump protein uptake. Thus, first-generation antibiotics have encountered antibiotic resistance in a variety of clinical settings via natural processes (Bartlett et al., 2013). Antibiotic resistance has emerged as a major worldwide health concern, associated to high rates of mortality and morbidity. Multidrug resistance made Gram-positive and Gram-negative bacteria-related infections harder to treat, and standard antibiotics were no longer effective. Antibiotic resistance has had a negative impact on antibiotic efficacy in clinical settings both before and after antibiotics were introduced (Velez & Sloand, 2016). The first antibiotic resistance was recorded in 1930, when sulfonamides were introduced; it predicted the development of antibiotic resistance in the natural environment prior to the antibiotic era, but did not support

the existence of fatal resistant pathogens. The development of antibiotics and their use in other areas of life marked the beginning of the antibiotic era. Human activity resulted in the use of high amounts of harmful antibiotics, which significantly altered their inherent importance and accelerated the growth of antibiotic-resistant microorganisms. Antibiotic resistance is as old as the therapeutic use of antibiotics, and it has become widespread and resistant due to drug agents' capacity to inactivate their cell walls. Research suggests chemical modification of antibiotics to prevent cleavage by penicillinases (β-lactamases) (D'Costa et al., 2006). According to the researchers, a better understanding of how infectious disease-induced biochemical mechanisms and specific virulence strategies work opens new opportunities to attack and interact with critical pathogenicity variables or infectivity characteristics of bacteria without subjecting them to pressure from evolution to acquire resistance. Prior to human intervention, the cellular mechanism of antibiotic resistance included increased antibiotic use, genetic alteration of bacterial strains, and antibiotic resistance genes. They have also been released from natural genetic sources, swiftly spreading to infectious and commensal bacteria of several taxonomic groups.

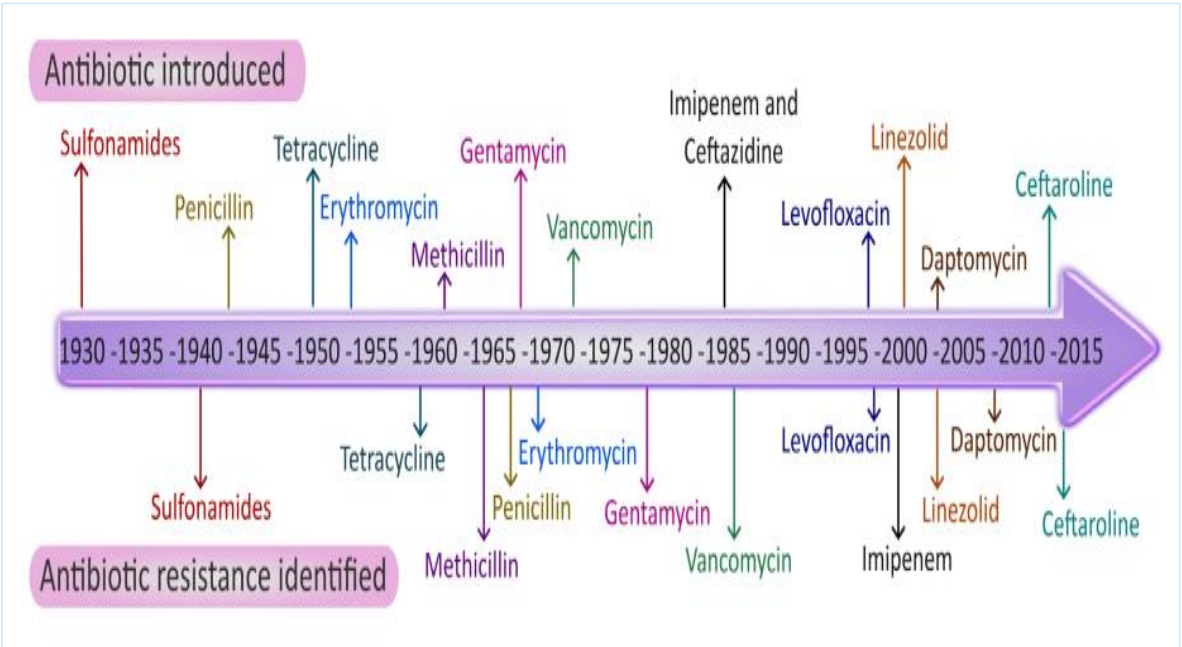

Figure 1.10. Timeline of antibiotic resistance compared to antibiotic development (Clatworthy et al., 2007; Ventola, 2015)

1.13 Virulence Genes in *Campylobacter jejuni*

Bacterial virulence factors are specialized molecules, structures, or mechanisms that allow bacteria to invade host tissues, evade or suppress immune responses, and cause disease. These factors are crucial to a bacterium's capacity to infect and proliferate within a host (Finlay & Falkow, 1997). *Campylobacter* virulence genes are *rack, racR, parC, gyrB, wlaN, virB11, cadF, ceuF, flaA, cdtA, cdtB, cdtC,* the *cdt* gene cluster*, ciaB, virB11,* and *galE*. Several virulence factors have been discovered as contributing to the pathogenesis of *Campylobacter* infections in humans, including a cytolethal-distending toxin (CDT) that targets the epithelial cell layer, inducing cellular distension and death in several cell lines (Guerry et al., 2012). The CDT activity is encoded by the *cdt* genes, which in *Campylobacter jejuni* are three contiguous or slightly overlapping genes known as *cdtA, cdtB*, and *cdtC*. *C. jejuni, C. coli, C. lari, C. fetus, and C. upsaliensis* all produce cytolethal distending toxins. Lior reported the first instance of *Campylobacter* isolates generating CDT in 1988.

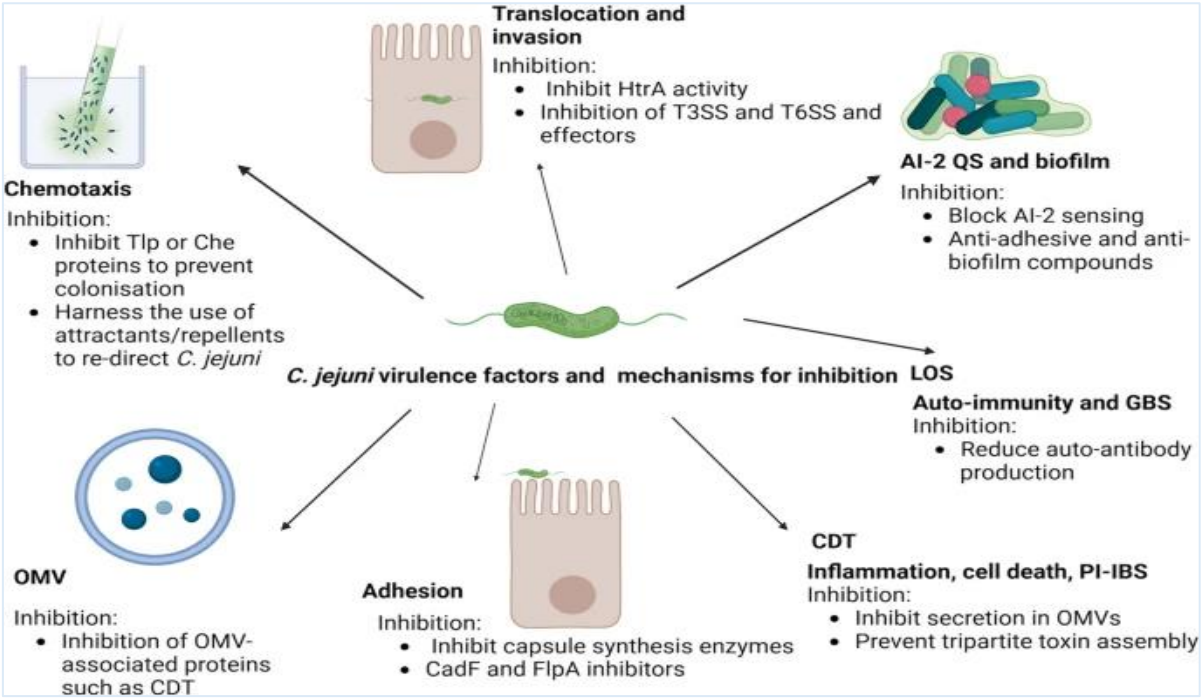

Figure 1.11. Summary of the central virulence factors of *C. jejuni* (Tikhomirova et al., 2024).

Pathogenic genes *flaA, cadF, rack* and *dnal* are responsible for adherence and colonization; *virB11, ciaß,* and *pldA* are pathogenic genes responsible for invasion; *cdtA, cdtB,* and *cdtC* are pathogenic genes responsible for toxin production; and *wlaN* was chosen as a gene that is

presumably involved in the expression of ganglioside mimics in Guillain-Barré syndrome (Datta et al., 2003). *Ceul*, another potential virulence gene in *Campylobacter jejuni*, encodes a lipoprotein (part of a protein-binding-dependent transport pathway for the siderophore enterochelin) (Clatworthy et al., 2007; Guerry et al., 2012). *C. jejuni* secretes the *Campylobacter* invasion antigen (CIA proteins). The M of the *Cia* protein ranges between 12.8 and 108 KDA. To date, just one secreted protein, *Cia*, has been identified (Konkel et al., 1999). The *C. jejuni Cia B* gene produces a 610-amino acid protein with a calculated molecular mass of 73,154 Da. While a co-focal microscopy analysis of *C. jejuni* infected cells demonstrated that *CiaB* is translocated into the cytoplasm of host cells, the precise role of *CiaB* remains uncertain. The *C. jejuni ciaB* null mutant cannot secrete the entire pool of *Cia* proteins.

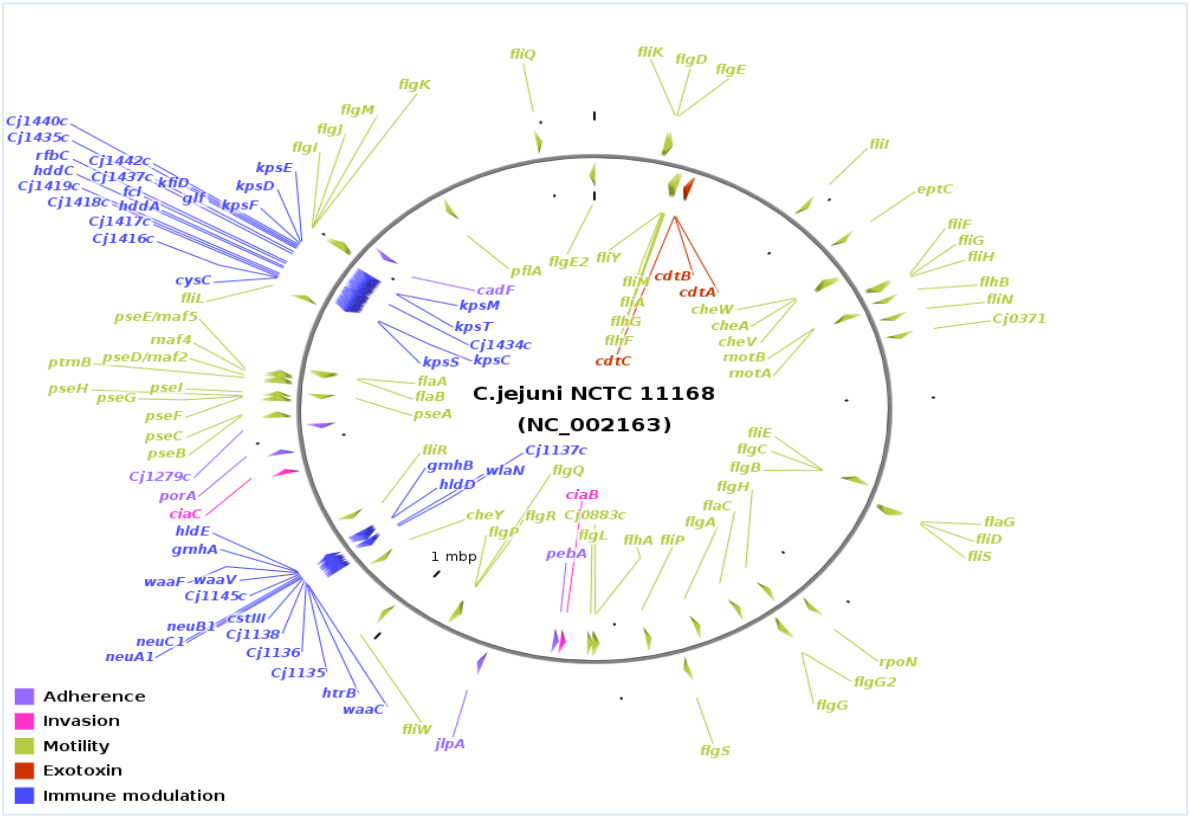

Figure 1.12. Genomic location of virulence-related genes in *Campylobacter jejuni* (Image courtesy- VFDB).

1.14. Aims of the study

1.14.1. *General objective:*

Characterization of AMR patterns and virulence genes of *Campylobacter jejuni* strains isolated from clinical, poultry and wastewater interfaces in Bangladesh.

1.14.2. *Specific objectives:*

- Isolation and identification of *Campylobacter jejuni* strains by biochemical tests and PCR.
- Determination of antibiotic-resistant patterns of *Campylobacter jejuni* in the multisource interface using disc diffusion method and E-test
- To evaluate the prevalence of virulence genes in *Campylobacter jejuni* isolated from diverse sources through molecular techniques (PCR).

Chapter 2

Methods and Materials

2.1. Isolation and identification of *Campylobacter jejuni*

A total of fifteen *Campylobacter* stock isolates were used in this study. A group of fifteen stock isolates that had been cultured included *Campylobacter jejuni* isolated from clinical, poultry and wastewater samples. Stock samples were cultured at the CHROMagar plate and kept in an Oxoid^{TM} AnaeroJar^{TM} (Thermo Scientific) with a microaerobic condition by using CampyGen (it comprises a paper gas-producing sachet and a plastic pouch with a closing clip). The ascorbic acid in the paper sachet interacts with air to create microaerobic conditions, which are ideal for the development of microaerophilic organisms). The jar was kept for 48 hours at 42°C for incubation and the remaining part of the stock sample was stored at -80°C. *Campylobacter* colonies were sought by non-hemolytic, coccus, red dot, flat, and wet at CHROMagar plate. Positive colonies were inoculated in Brain Heart Infusion Broth (BHIB) and incubated for 48 hours at the same condition.

2.2. Molecular Characterization

2.2.1. DNA Extraction by Boiling Method

The colony which was positive for biochemical tests was subjected to DNA extraction by boiling method for PCR to identify 16S rRNA for confirmation of genus *Campylobacter* and random gene for confirmation of species *jejuni*. Random gene is absent from all species of *Campylobacter* other than *Campylobacter jejuni* which is subjected to identify *C. jejuni* species. Samples were taken from BHI and MH broth which were incubated for 48 hours at 42°C in a jar having a microaerobic condition (10% CO2, 5% O2, and 85% N2) by CampyGen Sachet. DNA extraction samples were stored at -80°C in 25% glycerol for further validation.

Procedure: A total of 180 µl autoclaved distilled water was added with 20 µl bacterial culture from Brain Heart Infusion broth into a microcentrifuge tube in a total of 200 µl volume. The mixture was gently spin to be a homogenized mixture. The mixture was centrifuged at 11,000 rpm for 10 minutes and discarded the supernatant. After that, the pellet was suspended by 200 µl TE buffer which was subjected to boil for 10 minutes which helps to denature proteins (cell

wall), and extract DNA from spots. After that, the mixture was cooled at -20°C for 4 minutes which can improve the efficiency of bacterial DNA extraction. After that, the mixture was again centrifuged at 11,000 rpm for 10 minutes. Bacterial DNA is released into the aqueous phase so that supernatant was collected at 180 µl for PCR confirmation by 16s rRNA and random gene.

2.2.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a biochemical technique that is used to amplify a single copy of DNA into thousands to millions of copies of DNA by several orders of magnitude of a distinct sequence of DNA which can be visualized by bands on the agarose gel.

Master Mix Preparation

Master mix composition was prepared by adding Nuclease free water, 5x PCR buffer, 25mM Mgcl2, 10 mM dNTPs, 10 nM Primer (forward and reverse) and taq polymerase with a total volume of 22µl. Then, 3µl template DNA was added with the master mix which would be a total of 25µl volume.

Procedure for 16s rRNA and Random gene: Bacterial DNA was extracted as previously described. The assay was developed using a combination of newly designed and previously reported primers in consideration of the size of the PCR product and annealing temperature. Two primers were designed from sequences (Table 2.1). PCR products with a length of 833 bp and 773 bp respectively for, the genus *Campylobacter* (16s rRNA) and species *jejuni* (Random). DNA amplification was performed in a BioRad PCR thermal cycler. The DNA preparations described above were used as template DNA in PCR reactions containing Master Mix. The PCR reactions were performed in a thermocycler according to the following schedule: for each cycle, the sample was denatured at 95°C for 5 minutes followed by 30 seconds at the same temperature. Then the temperature dropped to 59°C (16s rRNA) and 61°C (Random) for 1 min for annealing then the temperature rose slowly to 72°C for 1 min. The temperature remains constant for 10 min and drops to 4°C for an infinite time. The cycle had to be carried out for 31X(16s rRNA) and 29X(Random) cycles.

Table 2.1. Two sets of primers for gene amplification. The length of the amplified DNA fragments is also indicated.

Species	Size (bp)	Target gene	Sequence (5' to 3')	Anneal-ing Temp.
Genus *Campylo bacter*	833	16S rRNA	F5'-ATCTAATGGCTTAACCATTA-3'	59°C
			R5'-GTAACTAGTTTAGTATTCCGG-3'	
Species *jejuni*	773	Random	F5'-CATCTTCCCTAGTCAAG CCT-3'	61°C
			R5'-AAGATATGGCTCTAGCAAGAC-3'	

2.2.3. Gel Electrophoresis

Gel electrophoresis is a scientific technique for separating DNA using an electrical charge. Molecules are propelled forward by the applied electric field. One end of the gel is positively charged, while the other end is negatively charged. As DNA is a negatively charged molecule, it may migrate from the negative end of the gel to the positive end.

Procedure: The 1.2% agarose was made by dissolving agarose powder in 100 ml 0.5M TBE and melting it in an oven for 2 minutes, gently swirling until no particles remained. Allow about 20 minutes for the gel to set. For visualization of PCR products, 10 µl of sample aliquots and 2.5 µl ladder were loaded. Power was turned on and the voltage control knob was set at 120 volts. The sample was run until 50% of the bromophenol blue migrated to the positive electrode end of the gel. Unplugged the power supply. The gel was withdrawn and put in a solution of ethidium bromide. It was permitted 30 minutes in the Staining solution. The gel was destained by soaking it in distilled water for 15 minutes. The gel was loaded into the Molecular Gel Doc imager and the software was run to capture a snap of the gel. Positive 16S rRNA, Random, Ceu gene-positive samples for Campylobacter jejuni species captured.

2.3. Antimicrobial Susceptibility Test by Modified Kirby-Baur Method

Bacterial susceptibility to antimicrobial agents was determined by the disc diffusion methods as recommended by The National Committee for Clinical Laboratory standards, 1900 with commercial antibiotics discs (Oxoid, Basingstoke, United Kingdom). The antibiotics discs used

in this study are given in the table below. The measurement zone of a diameter range of disc diffusion method is followed which is described by Taradon Luangtongkum and Teresa Y. Morishita, 2006.

Table 2.2. Breakpoints of disk diffusion methods used to determine antimicrobial susceptibility of *Campylobacter* isolates.

Class of Antibiotics	Antibiotics	Code	Conc. (µg)	Zone Diameter(nearest whole mm)		
				S	I	R
Aminoglycosides	Amikacin	AK	30	≥17	15-16	≤14
	Gentamicin	CN	10	≥15	13-14	≤12
Cephalosporins	Cefixitin	CFX	30	≥23	15-22	≤14
	Ceftriaxone	CTX	5	≥19	16-18	≤15
	Cefuroxime	CXM	30	≥18	14-17	≤13
	Cefixime	CFM	10	≥23	20-22	≤19
Penicillins	Amoxicillin	AMC	30	≥22	23-25	≤26
	Ampicillin	AMP	30	≥18	15-17	≤14
Carbapenem	Imipenem	IPM	10	≥17	14-16	≤13
Quinolones	Ciprofloxacin	CIP	30	≥19	15-18	≤14
	Nalidixic Acid	NA	30	≥18	13-17	≤12
Macrolides	Azithromycin	AZM	1	≥21	16-20	≤15
	Erythromycin	ERY	15	≥23	14-22	≤13
Sulfonamide	Sulfamethoxazole	SXT	15	≥23	14-22	≤13
Tetracycline	Tetracycline	TET	25	≥16	11-15	≤10
Chloramphenicol	Chloramphenicol	CHL	30	≥19	14-18	≤13

Procedure: By the standard method of inoculation, an inoculating needle was touched to a single well-isolated colony and inoculated into 2 ml of BHIB (Brain Heart Infusion Broth) & MHB (Muller-Hinton Broth). The broth cultures were then allowed to incubate at 42°C in a candle jar for 24 hours to obtain a young culture. The turbidity of actively growing broth cultures was then adjusted to a McFarland 0.5 standard (3×10^8 CFU/ml). To inoculate the agar medium, a sterile, nontoxic cotton swab was dipped into the standardized suspension, and the excess broth was purged by pressing and rotating the swab firmly against the inside of the tube

above the fluid level. The swab was then streaked evenly in three directions over the entire surface of the blood agar plate to obtain uniform inoculums. The final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for 3 to 5 min before the discs were applied. Antibiotic-impregnated discs were gently pressed down onto the blood agar with forceps to ensure complete contact with the agar surface. Within 15 min after the discs were applied, the plates were inverted and placed in an incubator at 42° C. After 16 to 18 hours of incubation, the plates were examined, and the diameters of the zones of complete inhibition were measured to the nearest whole millimeter by a ruler. The zone diameters for individual antimicrobial agents were then translated into susceptible, intermediate, or resistant categories according to the interpretation table (supplied by Oxoid Limited, England).

2.4. Determination of Minimum Inhibitory Concentration (MIC) by E-test

Minimum inhibitory concentration (MICs) to Tetracycline, Erythromycin, and Ciprofloxacin were determined or estimated with the use of the E-test strips (AB-Biodisc, Solna, Sweden) according to guidelines from the National Committee for Clinical Laboratory Standards (NCCLS). The use of minimum inhibitory concentration range was performed by Taradon Luangtongkum and Teresa Y. Morishita, 2006.

Procedure: By the standard method of inoculation, an inoculating needle was touched to a single well-isolated colony and inoculated into 2 ml of BHIB (Brain Heart Infusion Broth) & MHB (Muller-Hinton Broth). The broth cultures were then allowed to incubate at 42°C in a candle jar for 24 hours to obtain a young culture. The turbidity of actively growing broth cultures was then adjusted to a McFarland 0.5 standard (3×108 CFU/ml). To inoculate the agar medium, a sterile, nontoxic cotton swab was dipped into the standardized suspension, and the excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then streaked evenly in three directions over the entire surface of the blood agar plate to obtain uniform inoculums. The final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for 3 to 5 min before the discs were applied. Antibiotic-impregnated discs gently pressed down onto the blood agar with forceps to ensure complete contact with the agar surface. Within 15 min after the discs were applied, the plates were inverted and placed in an incubator at 42° C. After 16 to 18 hours of incubation, the plates were examined, and the diameters of the zones of complete inhibition were measured to the nearest whole millimeter by a ruler. The zone diameters for individual

antimicrobials agents were then translated into susceptible, intermediate, moderately susceptible, or resistant categories according to the guidelines provided by the National Committee for Clinical Laboratory Standards.

Table 2.3. Determination of Minimum Inhibitory Concentration by E Test used to determine antimicrobial susceptibility of *Campylobacter* isolates.

Antimicrobial Agent	Test Range(mg/ml)	MIC Breakpoint(µg/ml)		
		S	I	R
Ciprofloxacin	(0.002-32)	≤0.5	1-4	≥8
Erythromycin	(0.016-256)	≤4	8	≥16
Tetracycline	(0.016-256)	≤1	2	≥4

***Key Points: S=Sensitive, I=Intermediate, R=Resistant**

2.5. Procedure for Virulence Genes Identification Amplicon

The sequence of the primers and the DNA preparations described above were used as template DNA in PCR reactions containing Master Mix. The PCR reactions were performed in a thermocycler according to the following schedule: for each cycle, the sample was denatured at 95°C for 5 minutes followed by 30 seconds at the same temperature. Then the temperature dropped to 53°C (*flaA* gene) for 1 min for annealing then the temperature rose slowly to 72°C for 1 min. The temperature remains constant for 10 min and drops to 4°C for an infinite time. The cycle had to be carried out for 30X cycles.

Table 2.4. PCR primers for virulence genes

Target gene	Primers	Sequence(5' to 3')	Annealing T (°C)	Product (bp)
flaA	flaA 494	TACCGAACCAATGTCTGCTCTGATT	53	855
	flaA 664	AATAAAAATGCTGATAAAACAGGTG		
gyrB	gyrB F	ATGGCAGCTAGAGGAAGAGA	60	960
	gyrB R	GTGATCCATCAACATCCGCA		
cdtC	cdtC 192	CGATGAGTTAAAACAAAAAGATA	47	672
	cdtC 351	TTGGCATTATAGAAAATACAGTT		
racR	racR 25	GATGATCCTGACTTTG	45	584
	racR 593	TCTCCTATTTTTACCC		
wlaN	wlaN-DL39	TTAAGAGCAAGATATGAAGGTG	46	672
	wlaN-DL41	CCATTTGAATTGATATTTTTG		
parC	parC F	TGGGATCCAAACCTGTTCAGCGCC	65	395
	parC R	CGGAATTCGTGGTGGTGCCGTTAACAA		
cdtA	DS 15	ACACTCCATTTGCTTTCTG	49	370
	DS 18	CCTTGTGATGCAAGCAATC		

Chapter 3

Results

3.1. Bacterial Strains Isolation

Samples of fifteen *Campylobacter jejuni* from stock were cultured into CHROMagar and Brucella agar culture plate and colonies were grown at morphological characteristics of coccus, red dot, wet-like colonies in CHROMagar plate and transparent, mucoid, and grayish in Brucella agar plate. Colonies from the culture plate were inoculated at Brain Heart Infusion (BHI) broth and *Campylobacter* appeared as hazy in broth despite the control being transparent.

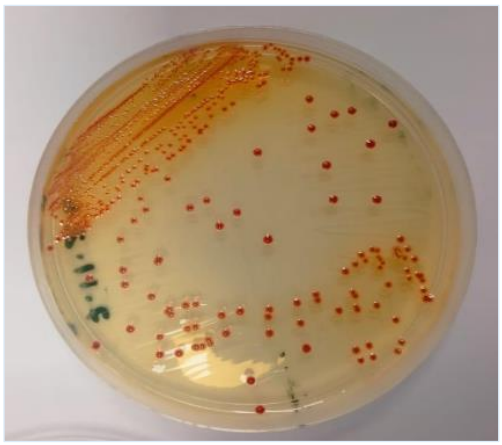

Figure 3.1. *C. jejuni* growth in medium (Chrom agar).

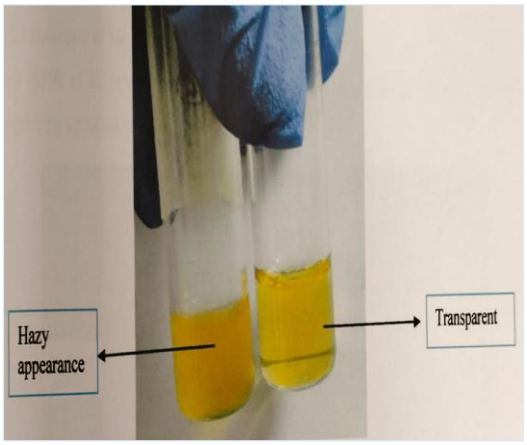

Figure 3.2. *C. jejuni* grown on BHI broth.

3.2. Phenotypic Characterization

3.2.1. Biochemical Tests

Oxidase test

After completion of incubation of samples in CHROMagar, Oxidase test carried out on different suspected colonies even several colonies on one plate. In chicken samples, all colonies were performed as oxidase positive, and almost all colonies of water samples were found

Figure 3.3. Oxidase test.

as oxidase positive. Oxidase positive organisms were given a characteristic deep purple or blue color with Kovac's oxidase reagents.

Catalase Test

Catalase test was also carried out on oxidase positive colonies grown on the mCCDA plate. We found catalase positive from 15 different samples. These samples were primarily suspected as *Campylobacter* and used for PCR confirmation.

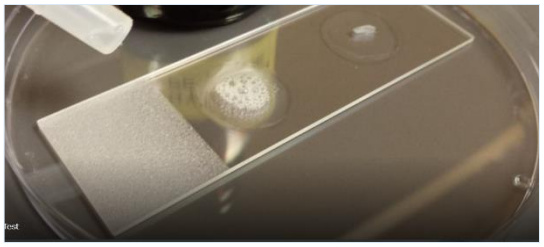

Figure 3.4. Catalase test.

Hippurate Hydrolysis Test

Hippurate hydrolysis test is carried out only those types of colonies which were performed as both catalase and oxidase test positive. Hippurate Hydrolysis is the most important phenotypic characterization of *Campylobacter jejuni*. Hippurate Hydrolysis methods were used to differentiate *C. jejuni* with other *Campylobacter* species.

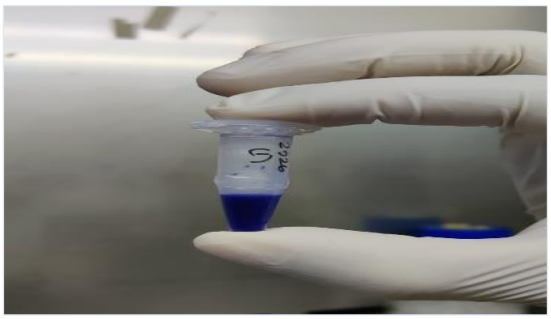

Figure 3.5. Hippurate Hydrolysis test.

3.3. Molecular Characterization

Samples from BHI broth were subjected to PCR confirmation for *Campylobacter jejuni*. The presence of 16S rRNA was used to identify *Campylobacter spp.* While random gene was used to confirm the *Campylobacter jejuni* species by PCR. Random gene was unique for the *C. jejuni* species.

3.3.1. Detection of 16s rRNA and Random gene by PCR

A polymerase chain reaction (PCR) was performed using the previously described primers and protocol with minor modifications. Two genes were selected for detection of the genus *Campylobacter* and the species *jejuni*. They were respectively 16S rRNA gene and Random gene. The sequence and the origin of the two sets of primers used for gene amplification are indicated in table 2.1.

16S rRNA of *Campylobacter* species was detected using the primer set CeCj609-F and CoCj1442-R by amplifying a product of 833bp. The positive control for *C. Jejuni* 16S rRNA (833bp) was in lane 2, whereas the negative control for *C. jejuni* was in lane 1. Lane 10 had the 50 bp ladder, whereas the remaining lanes contained 833 bp amplicon products.

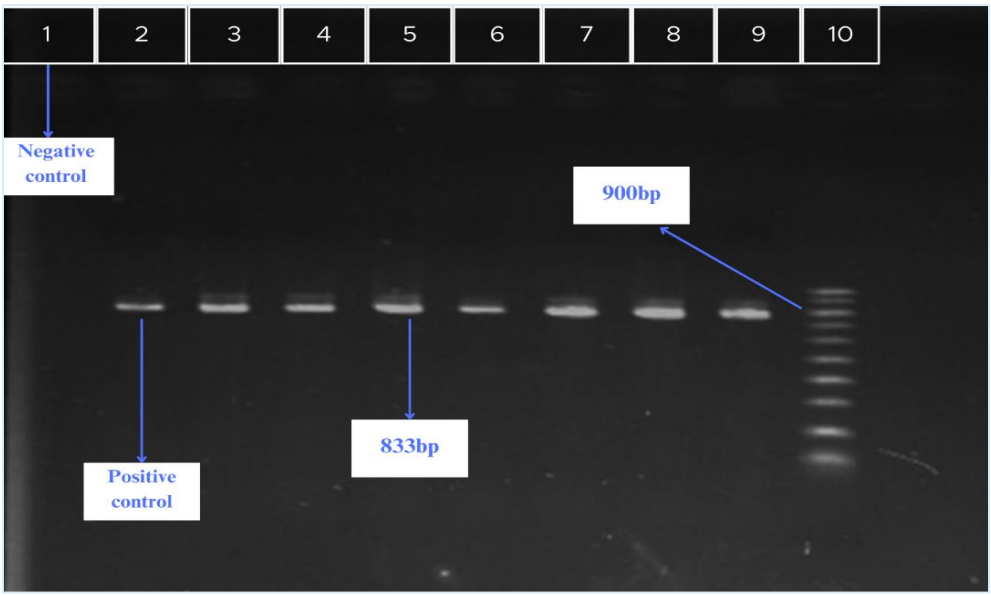

Figure 3.6. Agarose Gel electrophoresis showing PCR products of 16s rRNA amplicon for *Campylobacter* spp.

Random gene was amplified by PCR and detected by a 773 bp product with a set of primer. Figure 3.6 of agarose gel electrophoresis showed PCR amplicon products for random gene. Lane 2 was the positive control (773bp) and lane1 was the negative control of *C. jejuni*. DNA ladder (50 bp) was at lane 10 and rest of the lanes showed 773bp PCR amplicon products.

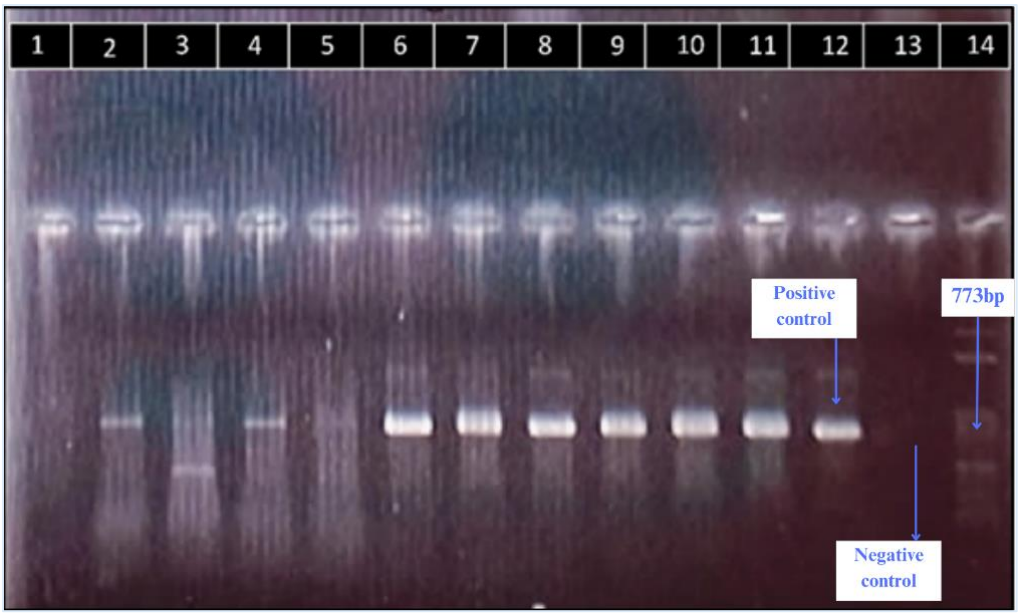

Figure 3.7. For further confirmation of the *C. jejuni* species, random gene was amplified by PCR and shown on agarose gel electrophoresis.

3.4. Antibiotic Susceptibility Test

An E-test method was used to establish the minimum inhibitory concentration (MIC) of *C. jejuni* isolates to 3 antimicrobials (erythromycin, ciprofloxacin, and tetracycline) using the blood agar susceptibility plates. The strains were sub-cultured on chrom agar at 42◦C for 48h under microaerobic conditions. The minimum inhibitory concentration of the antimicrobial agents was determined using BHI Broth. The antimicrobials and cut-off values used for the interpretation of the MIC results were by CLSI.

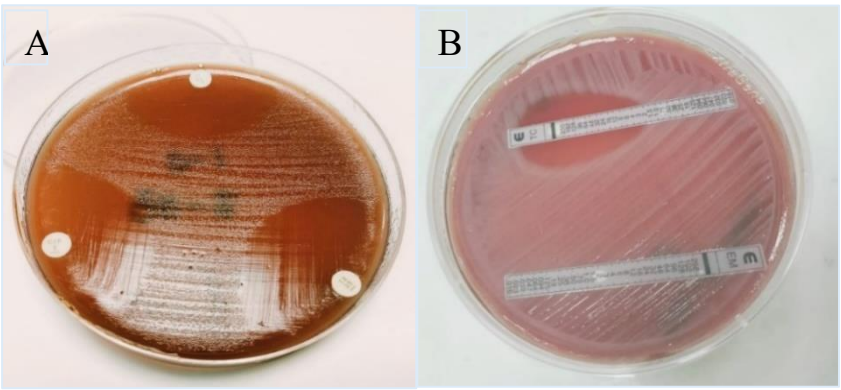

Figure 3.8. A. AST of *Campylobacter jejuni* in Blood agar plate; B. MIC confirmed by E-Test

3.4.1. AMR profile of 15 MDR *C. jejuni* strains

The results of the AMR profile of 15 multi-drug resistant *Campylobacter jejuni* strains showed that there was high 100% resistance to erythromycin and ciprofloxacin. To a lesser extent, *C. jejuni* was 46.66% resistant to chloramphenicol and 26.66% to imipenem. Extremely low levels of 6.66 % resistant to amikacin whereas no isolates were 100% susceptible to any antibiotic. However, the 15 *C. jejuni* isolates, all are multi-drug resistant (MDR). In this research, *C. jejuni* showed different antibiotic resistance patterns (figure 3.8).

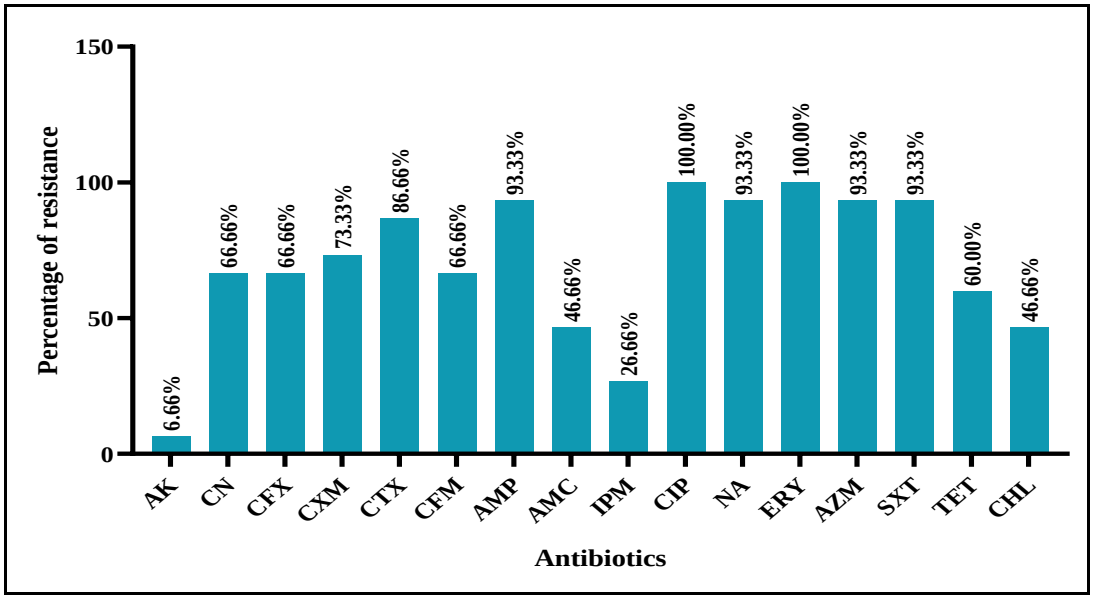

Figure 3.9. AMR profile of 15 multi-drug resistance *C. jejuni* strains.

3.4.2. AMR profile of *C. jejuni* among sources

The results of antimicrobial resistance of the *Campylobacter jejuni* isolates are shown in figure 3.9. The highest phenotypic resistance in AST displayed by *C. jejuni* isolates from wastewater isolates complete resistance (100%) was observed against eight antibiotic belongs to six different classes, while resistance to amikacin (20%) was the least observed. In the case of clinical isolates, the highest resistance (100%) was observed against five antibiotics belonging to four classes of antibiotic, while complete susceptibility to amikacin was observed. In chicken isolates, the highest resistance (100%) was observed against six antibiotics which belong to five different antibiotics class, while complete susceptibility to amikacin was observed. Overall, strains from three sources were 100% resistant to ciprofloxacin, erythromycin, and sulfamethoxazole, 93.3% resistant to nalidixic acid and azithromycin and 86.6% resistant to

ceftriaxone. There are least statistical differences in the resistance rates between strains recovered from clinical, poultry, and wastewater isolates. In case of wastewater significantly more isolates were 100% resistant to six drug classes (aminoglycosides, cephalosporins, penicillin, quinolones, macrolides, and sulfonamides) than those obtained from poultry and clinical isolates. The same pattern was observed for poultry and clinical isolates respectively where were 100% resistant to four drug classes (penicillin, quinolones, macrolides, and sulfonamides) and four drug classes (cephalosporins, quinolones, macrolides, and sulfonamides). A small number of isolates, irrespective of the origin, was less 20% resistant to amikacin and imipenem.

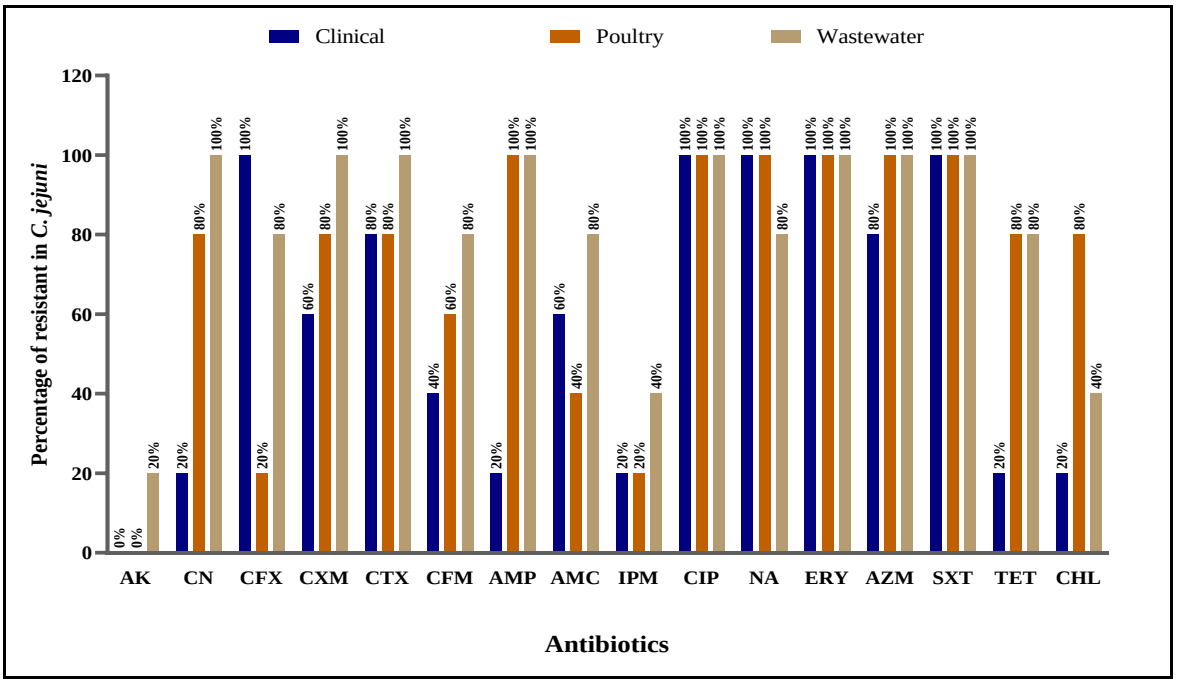

Figure 3.10. AMR profile of *C. jejuni* among sources.

3.5. Determination of Minimum Inhibitory Concentration (MIC) by E-test

The E-test was done for fifteen samples to check the MIC of *Campylobacter* against antibiotics such as Erythromycin, Tetracycline, and Ciprofloxacin. All isolates interpreted as resistant according to E-test and resistant according to AST were similar. No minor or major errors were observed. Additionally, the correlation between methods following logarithmic transformation was good and highly significant for all the tested antibiotics . The MIC distribution in all *C. jejuni* isolates was assessed for erythromycin, tetracycline, and ciprofloxacin, as shown in

Table 3.1. Among strains, three antimicrobials demonstrated a high resistance rate in MIC values. All isolates showed resistance rates of erythromycin (100%), tetracycline (73.33%), and ciprofloxacin (100%). These highly resistant strains were recovered from diverse sources.

Table 3.1. Antimicrobial susceptibility pattern of *Campylobacter. Spp*: comparison between E-test and the disk diffusion methods.

Sample ID	Erythromycin		Tetracycline		Ciprofloxacin	
	Zone Diameter Dose (µg) (15)	MIC (mg/ml) (0.016-256)	Zone Diameter Dose (µg) (30)	MIC (mg/ml) (0.016-256)	Zone Diameter Dose (µg) (5)	MIC (mg/ml) (0.002-32)
1	7^R	>256	15^R	>256	10^R	1.5
2	7^R	>256	14^R	>256	15^R	4
3	7^R	>256	14^R	24	7^R	>32
4	7^R	16	12^R	24	7^R	6
5	7^R	<.016	10^R	16	7^R	2
6	7^R	16	36^S	0.25	7^R	<0.002
7	7^R	>256	44^S	0.125	7^R	4
8	7^R	<.016	10^R	32	7^R	1.5
9	7^R	<.016	38^S	0.38	7^R	12
10	7^R	48	46^S	0.047	7^R	4
11	7^R	>256	10^R	12	14^R	4
12	7^R	>256	7^R	>256	7^R	>32
13	7^R	>256	7^R	>256	7^R	>32
14	7^R	>256	7^R	>256	7^R	>32
15	7^R	>256	15^R	1.5	7^R	>32

***Key Points: S=Sensitive, R=Resistant**

3.6. Correlation between E-test and disk diffusion method

The scatterplots in Figure 3.11 indicate that as the zone of inhibition increases, the MIC decreases, suggesting stronger susceptibility. The graphs demonstrate a strong inverse relationship between minimum inhibitory concentration (MIC) values and zone of inhibition for tetracycline, ciprofloxacin, and erythromycin, as determined by E-test and disc diffusion method. This parallel association demonstrates the dependability and consistency of both approaches in detecting antibiotic resistance. The results show that many isolates are resistant to erythromycin and ciprofloxacin. The synchronization of these two approaches verifies the precise profiling of antibiotic resistance. The findings underline the need for susceptibility testing in guiding appropriate treatment regimens, particularly for multidrug-resistant *Campylobacter jejuni* isolates.

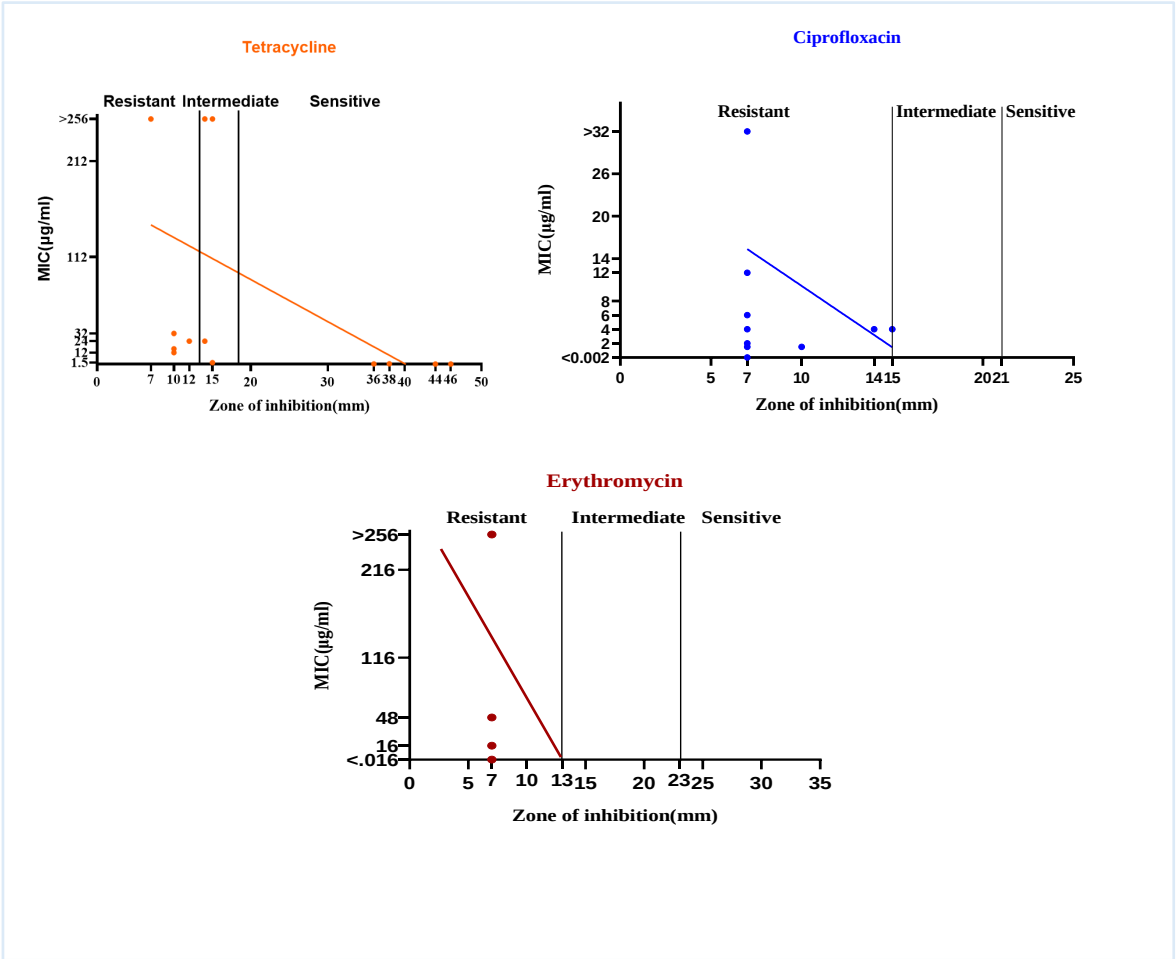

Figure 3.11. Antimicrobial susceptibility pattern of *Campylobacter jejuni spp.*: a comparison between E-test and disk diffusion method (Simple Linear Regression by using GraphPad).

3.7. Detection of Virulence genes of MDR *C. jejuni* strains by PCR

The *flaA* gene of *Campylobacter jejuni* was detected using the primer set *flaA* 494-F and *flaA* 664-R by amplifying a product of 855bp. On the agarose gel, PCR amplicon products were evident. The positive control for *C. Jejuni flaA* (855bp) was in lane 2, whereas the negative control for *C. jejuni* was in lane 1. Lane 9 had the 50 bp ladder, whereas the remaining lanes contained 855 bp amplicon products.

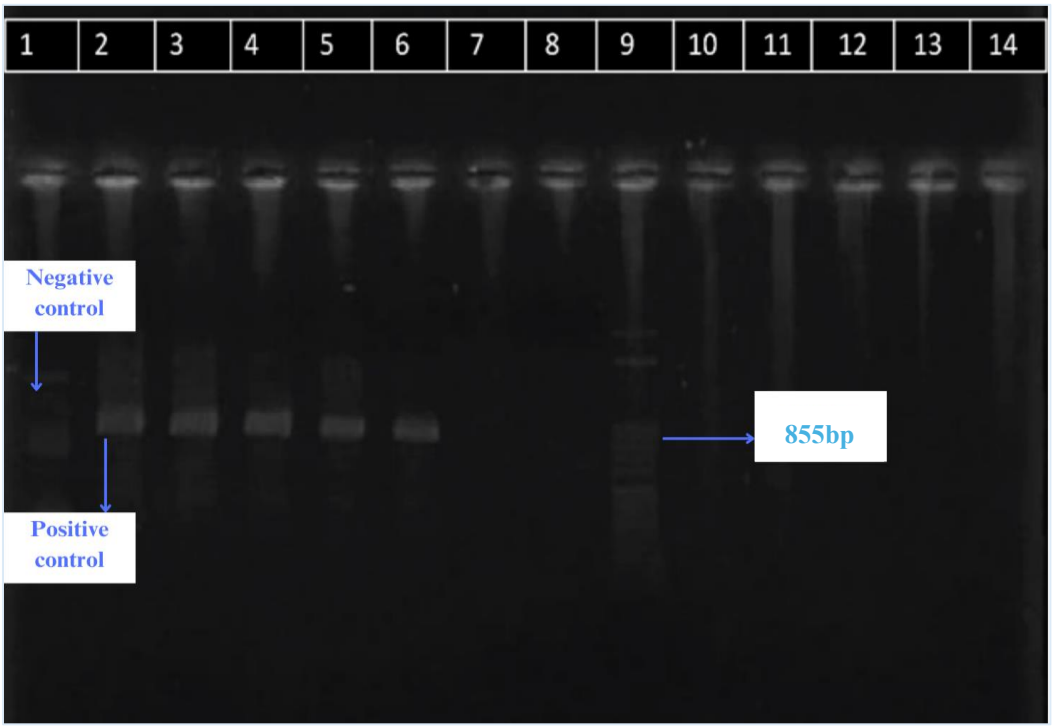

Figure 3.12. Agarose Gel electrophoresis showing PCR products of *flaA* amplicon for *Campylobacter jejuni*

3.8. Distribution of virulence genes in multiple interfaces

Figure 3.13 suggests a statistically significant difference in virulence factor distribution between at least one of the groups (Poultry, Wastewater and Clinical). The graph suggests that virulence genes are most prevalent in Clinical isolates, moderately present in Poultry isolates, and least prevalent in Wastewater isolates.

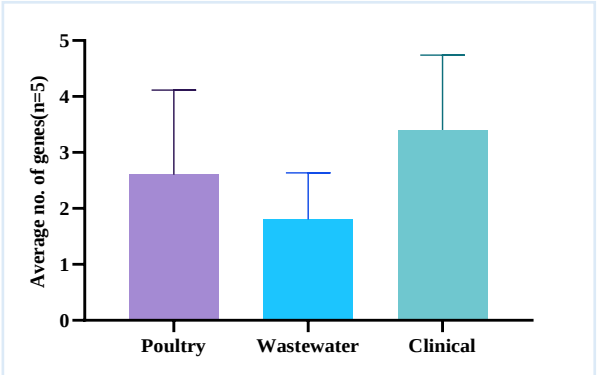

Figure 3.13. Average Virulence genes in each isolate of multiple interfaces.

Overall, the presence of virulence genes analyzed in *C. jejuni* isolates, especially for the genes associated with motility, adhesion, and colonization (*flaA, cdtA, cdtC, wlaN and racR*), DNA replication (*gyrB, parC*) of the bacteria. The putative virulence genes tested are *flaA, racR, cdtA*, *cdtC, gyrB, parC, and wlaN*. In the case of clinical isolates, the *flaA* gene is positive in 5 isolates (100%) whereas in wastewater isolates *parC* gene is positive in 5 isolates (100%). A group of three genes *racR, cdtA*, and *cdtC* are positive in 4 clinical isolates (80%) whereas only *cdtA* is positive in 4 chicken isolates (80%). Furthermore, the *wlaN* gene is found in 3 wastewater isolates (60%). The average presence of all virulence genes among sources are *cdtA*(53.3%), *flaA*(46.6%), *wlaN(40%), cdtC, racR,* and *parC* respectively (33.3%), and *gyrB*(20%).

Table 3.2. Virulence genes percentage in *C. jejuni* from multiple interfaces.

Gene	Clinical (n=5)	Wastewater (n=5)	Poultry (n=5)	Average (n=15)
flaA	100	0	40	46.6%
gyrB	0	20	40	20%
cdtC	80	0	20	33.3%
racR	80	0	20	33.3%
wlaN	20	60	40	40%
parC	0	100	0	33.3%
cdtA	80	0	80	53.3%

3.8. Heatmap of AMR profiles and Virulence genes of MDR *C. jejuni* strains

In figure 3.14 dark and light blue respectively indicate the presence and absence of a specific aspect. Virulence genes such as *flaA, gyrB*, and *cdtC* are inconsistently present across isolates, showing virulence potential, but some virulence factors are more uniformly present, implying greater pathogenicity. Antibiotic resistance profiles exhibit a variety of patterns, with some isolates demonstrating multi-drug resistance. Samples such as 2205425(clinical) include less virulence factors, indicating less pathogenic strains, but isolates such as BR-19(poultry) have extensive presence, indicating increased risk. Here, resistant(R); layer chicken (LR); broiler chicken (BR).

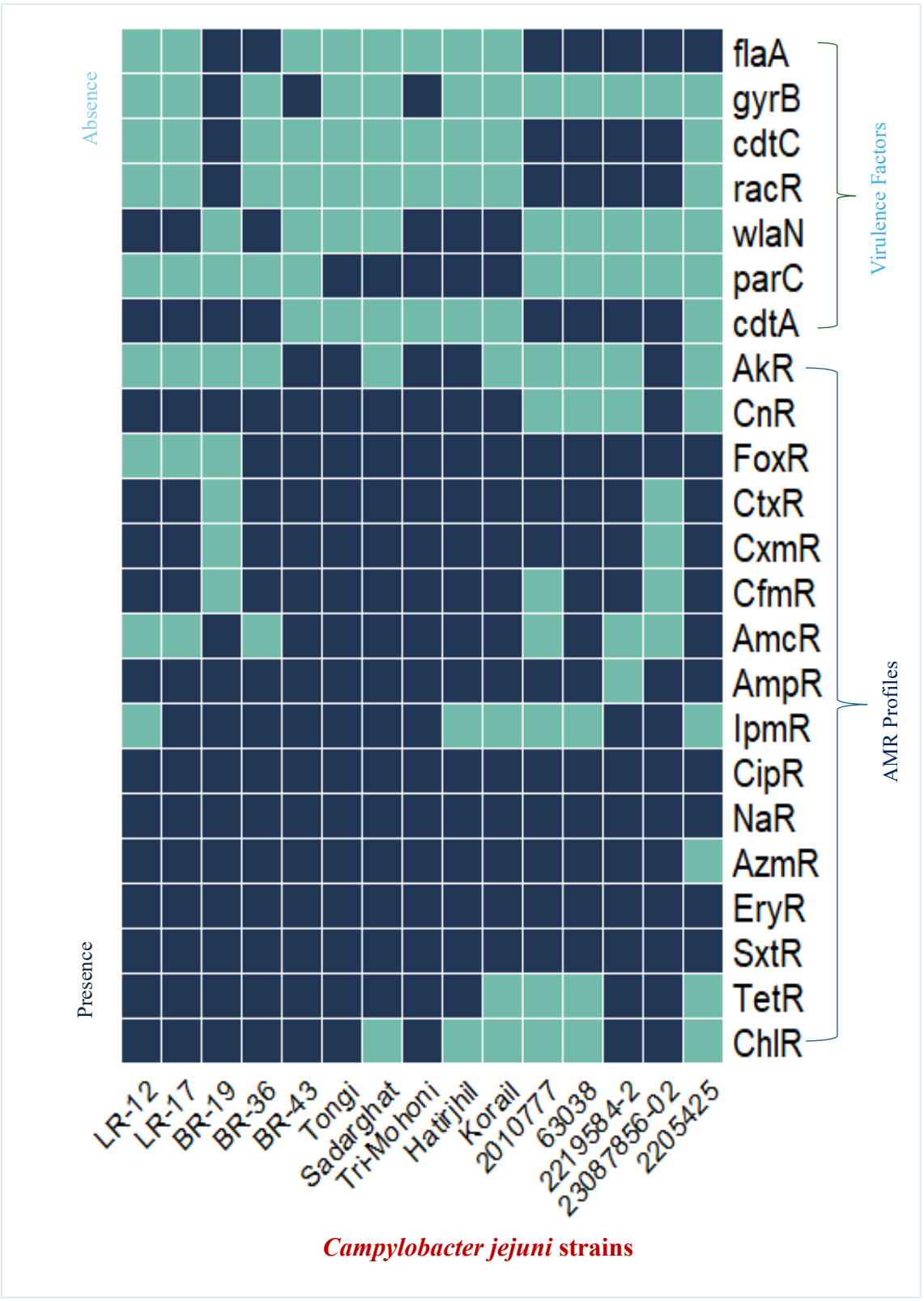

Figure 3.14. Heatmap of the AMR profile and virulence factors of 15 MDR *Campylobacter jejuni* strains.

Chapter 4

Discussion

Campylobacter species are involved in foodborne infections, and the emergence of multi-drug resistant (MDR) strains is becoming a serious health concern. Due to the limited studies on *Campylobacter* in Bangladesh, limited data is available on MDR patterns and pathogenic traits of local *Campylobacter* strains. In Bangladesh, most of the studies were limited to isolating *Campylobacter* species from patients with diarrhea. However sufficient data is not available yet on the prevalence of *Campylobacter jejuni* and MDR pattern of *C. jejuni* in multiple sources. Also, poultry and layer chicken in Bangladesh is harvested and consumed widely for meat and egg. Here recent developments in *C. jejuni* research, with a particular focus on MDR mechanisms and disease pathogenesis are highlighted. Hence, this study evaluated the multi-drug resistance-associated determinants and occurrence of virulence genes in *C. jejuni* species isolated from wastewater, humans, and livestock. Antibiotic resistance, which represents a global problem for public and animal health, is widely reported. The objective of this study was to identify and characterize MDR *C. jejuni* strains and detection of AMR resistance profile and virulence genes isolated from clinical (patients with gastroenteritis), livestock (poultry) and environmental (wastewater) obtained from different places in Dhaka, Bangladesh. These are primarily public water sources, and millions of low-income citizens of this massive and densely populated city live nearby. The spread of antimicrobial resistance is becoming a high concern due to the widespread use of antibiotics in the poultry industry. Our study results showed high rates of resistance to multiple antibiotics among *C. jejuni*. Other studies have also reported the high prevalence of such mechanism of resistance in *Campylobacter* isolates from various origins (Bolinger & Kathariou, 2017). The high spread of *Campylobacter* in the environment through clinical, livestock, and wastewater can easily be estimated from this study by whole genome sequencing.

Antibiotic Resistance in *Campylobacter* is gaining importance due to their increasing resistance to different important antibiotics. Nowadays fluoroquinolones and macrolides are the most used first-line antibiotics in the treatment of Campylobacteriosis. Thus, the treatment strategy for Campylobacteriosis has become more challenging today due to resistance. Unfortunately, we have found a very insufficient amount of study and data regarding the issue. That is why we aimed to see the current situation of the growing resistance of *C. jejuni* against mostly used

antibiotics and antibiotic susceptibility test was performed on fifteen *C. jejuni* isolates. The growing antibiotic resistance of *Campylobacter* isolates in humans, and animals via environment is rapidly becoming a major public health issue (Ghosh et al., 2013; Ghunaim et al., 2015; Shobo et al., 2016). Although gastroenteritis caused by *Campylobacter* is usually self-limiting, antibiotic treatment is recommended in protracted or bacteremic cases. The recommended antimicrobials for treating *Campylobacter* infections are macrolides (erythromycin, azithromycin, and clarithromycin), fluoroquinolones (ciprofloxacin), and tetracyclines (Pérez-Boto et al., 2014). However, resistance to these empirical medicines has been recorded in several countries (Garcia-Migura et al., 2014; Ghosh et al., 2013; Pérez-Boto et al., 2014; Redgrave et al., 2014; Shobo et al., 2016).

The highest phenotypic resistance in AST displayed by *C. jejuni* isolates from wastewater isolates 100% resistance was observed against eight antibiotics belonging to six different classes, while 20% resistance to amikacin was the least observed. In the case of clinical isolates, the highest 100% resistance was observed against five antibiotics belonging to four classes of antibiotics, while complete susceptibility to amikacin was observed. In poultry isolates, the highest 100% resistance was observed against six antibiotics which belong to five antibiotic different classes, while complete susceptibility to amikacin was observed. Overall, strains from three sources were 100% resistant to ciprofloxacin, erythromycin, and sulfamethoxazole 93.3% resistant to nalidixic acid and azithromycin, and 86.6% resistant to ceftriaxone. The E-test MIC determination method and the disk diffusion method agreed well in identifying antibiotic resistance in *C. jejuni*. The most frightening fact is that every single isolate we studied was multidrug resistant. Resistance to mostly used drugs and the presence of MDR pathogens in open public environments gives us the essence of serious public suffering. Overall, regression analysis highlights a strong correlation between the E-test and disk diffusion methods for all three antibiotics. Several studies, worldwide, have shown that the expression of genes involved in motility, colonization, invasion of epithelial cells, and production of toxins plays an important role in the development of *Campylobacter-associated* diseases (Dasti et al., 2010). In the current study, most of the isolates were shown to contain associated virulence genes related to the pathogen adhesion, colonization, and motility traits. Moreover, the *cdtA* and *cdtC* genes, necessary for the expression of the CDT toxin, were detected in some *C. jejuni* isolates. The putative virulence genes tested are *flaA, racR, cdtA, cdtC, gyrB, parC,* and *wlaN*. In the case of clinical isolates, *flaA* gene is positive in 5 isolates (100%) whereas in wastewater isolates *parC* gene is positive in 5 isolates (100%). A group of three genes *racR, cdtA, and cdtC* are positive in 4 clinical isolates (80%) whereas only *cdtA* is positive in 4 poultry isolates

(80%). Furthermore, the *wlaN* gene is found in 3 wastewater isolates (60%). The average presence of all virulence genes among sources are *cdtA* (53.3%), *flaA* (46.6%), *wlaN* (40%), *cdtC, racR,* and *parC* respectively (33.3%), and gyrB (20%). The prevalence was 40% for *wlaN* among the strains we studied, referring also to a high number of strains from uncomplicated gastroenteritis cases. Strains associated with *wlaN* are present only in isolates from cases with gastroenteritis. Thus, any correlation between the presence of *wlaN* in *C. jejuni* and its ability to trigger the manifestation of GBS in humans can hardly be made. The only conclusion that can be drawn, based on the present and previous data, is not sufficient for the pathogenesis of the autoimmune neurological syndrome. In this study, *flaA* and *racR* were selected as pathogenic genes responsible for the expression of adherence and colonization, *cdtA* and *cdtC* as pathogenic genes responsible for the expression of cytotoxin production, *parC* and *gyrB* as antibiotic resistance genes responsible for DNA replication in the gastrointestinal environment, while *wlaN* was selected as a pathogenic gene responsible for the expression of Guillain–Barre syndrome.

When looking at the relationship between the prevalence of virulence genes and antimicrobial resistance, our results showed that virulence genes are associated with resistant strains. The existence of positive or negative associations between antimicrobial resistance and virulence genes in bacteria has been shown but is still controversial in the *Campylobacter* genus (Dasti et al., 2010; Lehtopolku et al., 2010). Indeed, some studies showed an in vitro increased invasion of resistant strains as compared to susceptible ones, while others described the tendency of susceptible strains to cause more severe infections than resistant strains (Feodoroff et al., 2009). Nevertheless, further investigations should be conducted to explore more in-depth the relationship between the pathogenic traits and the antimicrobial resistance in *Campylobacter* strains.

Control and prevention of campylobacteriosis in humans requires knowledge of transmission routes, antibiotic resistance profiles, and virulence capacities of isolates. The results obtained in the present study showed the presence of *C. jejuni* species in the studied communities displaying varying degrees of resistance, especially to the empirical drugs used for the treatment of *C. jejuni* infections. Also, while strains isolated in the study carried virulence genes, the detection rate of these genes was higher in the human and chicken samples than in the water isolates. Transmission dynamics revealed that *C. jejuni* infection from the studied communities might have been acquired through the consumption of poor sanitation, and contaminated food and water.

Chapter 5

In conclusion, this study evaluated the multi-drug resistance (MDR) and occurrence of virulence genes in *Campylobacter jejuni* species isolated from wastewater, human, and livestock isolates. It might be a milestone study of *C. jejuni* and its resistance nature to most antibiotics in Bangladesh, particularly in the first-line drugs such as macrolide and fluoroquinolone for campylobacteriosis treatment. Further analysis of the whole genome sequencing is needed to determine how MDR *C. jejuni* in clinical settings is related to the livestock and environment-related *C. jejuni.* The metagenomics approach needs to address multisource resistome profiles and their transmission dynamics at the clinical- livestock - environment interfaces.

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