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MOLECULAR ANALYSIS OF *CAMPYLOBACTER JEJUNI* VIRULENCE GENES FROM CLINICAL ISOLATES IN QATAR

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By

Idil Ibrahim Aigha

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COMMITTEE PAGE

The thesis of **Idil Ibrahim Aigha** was reviewed and approved by the following:

We, the committee members listed below accept and approve the Thesis/Dissertation of the student named above. To the best of this committee's knowledge, the Thesis/Dissertation conforms the requirements of Qatar University, and we endorse this Thesis/Dissertation for examination.

Name: Dr. Marawan Abu Madi

Signature _____ Date 22/6/2014

Name: Dr. Sanjay H. Doiphode

Signature Dr. S. Doiphode Date 22/6/2014

Name: Dr. Gheyath K. Nasrallah

Signature _____ Date 22/6/2014

Abstract:

Background: *Campylobacter jejuni* is the main cause of gastroenteritis worldwide. Symptoms of *C. jejuni* infections range from asymptomatic to bloody diarrhea. Previous studies showed a strong association between diversity in presence of bacterial virulence factors, and the different outcomes of symptoms. *C. jejuni* relies on a set of virulence factors including: *Campylobacter* invasion protein B (CiaB), fibronectin-binding outer membrane protein (CadF), cytolethal distending toxin B (CdtB), Clp ATP dependent protease (ClpP) and HtrB protein. The aim of this project was to study the association between prevalence of the aforementioned genes, the antimicrobial resistance patterns of the isolates, and the demographic data of patients.

Methodology: A total of 174 archived isolates of *C. jejuni* were obtained from stool samples of symptomatic patients in the Microbiology Laboratory at Hamad Medical Corporation (HMC). Isolates were revived using selective media and DNA was extracted from positive colonies. *C. jejuni* isolates were screened by real-time PCR for the presence of five of virulence-associated genes. Isolates were also tested for the antimicrobial susceptibility to common antimicrobial agents used in HMC including: chloramphenicol, ciprofloxacin, erythromycin and gentamicin. Demographic data such as age, gender and nationality were also collected from the medical charts of subjects included in this study.

Results: 75% of the isolates were obtained from patients younger than 5 years old. 60.3% of the participants were males and 57% of the subjects were

from the Arabian Peninsula. All the virulence genes were detected with different percentages. There was a significant association between gender (male) and presence of two of the virulence genes *cadF* and *clpP* (*P* value: 0.022 for both). No clear associations were found between presence of the remaining virulence genes and demographic data; or between antimicrobial susceptibility patterns with age, gender and nationality. Isolates were highly sensitive to all antimicrobial agents except ciprofloxacin, where high resistance was observed.

Conclusion: Gender (male) was strongly associated with presence of *cadF* and *clpP* genes. Other correlations between prevalence of virulence genes, antimicrobial susceptibility and demographic data were found to be insignificant. Further investigation on the characteristics *Campylobacter jejuni*, and how they induce different symptoms and responses to their infection are needed.

Table of Contents

List of Tables

List of Abbreviations:

C. jejuni: *Campylobacter jejuni*

CadF: Fibronectin-binding outer membrane protein

CdtB: Cytolethal distending toxin B

CiaB: *Campylobacter* invasion protein B

ClpP: Clp ATP dependent protease

GBS: Guillain-Barre syndrome

HMC: Hamad Medical Corporation

PCR: Polymerase Chain Reaction

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Chapter 1 - Introduction

Campylobacter jejuni causes gastric illnesses and can in some rare cases cause some serious consequences. The emergence of this bacterial species as a significant causative agent of human disease and its ability to show antimicrobial resistance has made *C. jejuni* a serious public health concern. The ability of *C. jejuni* to colonize and survive in wide variety of animal species and habitats make them extremely difficult to control (Blaser et al., 1983).

Many studies have investigated the disease mechanisms of *C. jejuni* infection. The exact mechanism of campylobacteriosis remains unknown. However, it is agreed that the key role players in *C. jejnui* pathogenesity include colonization of the mucous layer of the intestine, adhesion and invasion of intestine epithelia, and finally the production of one or more cytotoxins that are usually associated with the severity of the symptoms (Ketley, 1997).

The variation in the pathogenicity among strains is a common feature in enterpathogens. *C .jejuni* strains have shown diversity in pathogenecity traits and factors including adhesion, invasion and toxicity. Several studies have attempted to associate the presence of known virulence-related genes to the disease phenotypes observed. Indeed there were strong associations between the presence of certain genes and the different outcome in phenotypic traits (Al-Mahmeed et al., 2006).

This dissertation discusses the background and literature of *C. jejuni*. It addresses the aspects of the human infection caused by this bacteria,

pathogenesis and virulent mechanisms. It will also discuss the antimicrobial susceptibility patterns of the isolates used in this study. Attempts to associate the presence of genes associated with pathogenesis, antimicrobial susceptibility testing results, and demographic data will also be discussed in details.

Hypothesis

In this study, we used clinical isolates of *C. jejuni* collected from the Microbiology Laboratory at Hamad Medical Corporation (HMC). Isolates were obtained and archived from stool samples of patients showing symptoms of abdominal pain, vomiting and diarrhea.

C. jejuni relies on a set of virulence factors that are mainly involved in colonization and the severity of the symptoms. These factors include *Campylobacter* invasion protein B (CiaB), fibronectin-binding outer membrane protein (CadF), cytolethal distending toxin B (CdtB), Clp ATP dependent protease (ClpP) and HtrB protein. Several studies have also reported antimicrobial resistance of *C. jejuni* isolates to several antimicrobial agents.

To our knowledge, so far no studies have investigated the prevalence of virulence factors or antimicrobial resistance in *C. jejuni* strains circulating in Qatar. Therefore, we hypothesize that the clinical isolates collected from HMC harbor the virulence factors that are vital to their pathogenesis. We also hypothesize that some of these isolates will have antimicrobial resistance to the most commonly used antimicrobial agents for treating *C. jejuni* infection. These antimicrobial agents include erythromycin, chloramphenicol, ciprofloxacin and

gentamicin. Using the demographic data of the patients, we also hypothesize that there might be some associations between age, gender and nationality with the presence of these factors, or with the antimicrobial resistance patterns.

Aims

The aim of this project was to characterize *C. jejuni* strains isolated from symptomatic patients referred to HMC in Qatar. *C. jejuni* isolates from those patients were screened for the presence of five of the major virulence factors of these bacteria that are involved in the pathogenicity of campylobacteriosis. They were also tested for the antimicrobial susceptibility to antimicrobial agents used in HMC.

Objectives

A. Testing the presence of the gene for the following factors: *Campylobacter* invasion protein B (CiaB), fibronectin-binding outer membrane protein (CadF), cytolethal distending toxin B (CdtB), ATP dependent Protease (ClpP) and the lipid A acyltransferase protein HtrB.

B. Determine the antimicrobial susceptibility of the clinical isolates to the antimicrobials routinely used to control the infection in HMC, including erythromycin, chloramphenicol, ciprofloxacin and gentamicin.

C. Study the demographic data including (age, gender and nationality) and analyze whether or not there are any specific patterns or associations with objectives A and B.

Chapter 2 - Literature Review

Campylobacter jejuni is the leading cause of bacterial foodborne illness in humans around the world. It is even more prevalent than enteritis caused by *Salmonella* (salmonellosis) (Blaser, 1990) as it is responsible for approximately 400 million cases of entero*coli*tis annually worldwide (Nachamkin et al., 1998; van Dijk et al., 2012).

Chicken infection with *C. jejuni* is asymptomatic despite the high colonization rate and load (Ruiz-Palacios et al., 1981). In humans, however, the infection is symptomatic. The disease "campylobacteriosis" is caused mainly by the consumption of contaminated food products of chicken and sometimes unpasteurized milk (Silva et al., 2011). Symptoms can range from slightly loose stools to a severe bloody diarrhea. Immunocompromised hosts develop an inflammatory diarrhea which usually lasts from three to five days (Dasti et al., 2010). The cause of this different outcome of *C. jejuni* infection in humans versus chickens is not well understood due to the lack of suitable animal models to mimic human campylobacteriosis (Guerry, 2007).

Microbiology of *Campylobacter jejuni*

C. jejuni belong to the family *Campylobacteraceae* which include the genera: *Campylobacter, Arcobacter* and *Sulfurospirillum*. In the genus *Campylobacter*, there are 18 species, with some of these species considered pathogenic to humans (On, 2013; Vandamme and Goossens, 1992). *C. jejuni* is a small Gram-negative, non-spore forming, microaerophilic, thermophilic spiral

shaped bacterium with one or bipolar flagella (Penner, 1988). It has a relatively small genome (1.6–2.0 megabases) that may contribute to the inability of *C. jejuni* to ferment carbohydrates and degrade complex substances (Parkhill et al., 2000) and may contribute to their requirement of special growth conditions and media (Griffiths and Park, 1990). The optimal cultural conditions of *C. jejuni* ranges from 37°C to 42°C and they strictly grow in microaerophilic atmosphere that consists of 3-15% oxygen and 3-5% carbon dioxide (Ketley, 1997).

C. jejuni is known to have a chemoorganotrophic metabolism. The bacteria derive their energy from amino acids or from tricarboxylic acid cycle (Penner, 1988). Human diseases are mostly associated with catalase positive *C. jejuni* strains (Ketley, 1997).The majority of the strains have a negative reaction with methyl red acetone, positive for indole and hippurate hydrolysis, and reduce nitrate and nitrite (Sellars et al., 2002; Vandamme and Goossens, 1992).

The complete genome sequence of *C. jejuni* strains and its plasmids was a breakthrough that started a new era of *C. jejuni* research. This provided resources for a complete and detailed analysis of the pathogenic potential of this bacterium and understanding the molecular basis of the virulence factors (Parkhill et al., 2000).

Epidemiology

C. jejuni infections are extremely common worldwide, although exact figures are not available. There are around 2.4 million cases of *Campylobacter* enteritis in the United States every year which accounts for 5 to 7% of the total

cases of gastroenteritis (Blaser, 1990; Slutsker et al., 1997). Almost 77% of the recorded cases of foodborne illness in the United Kingdom during 2000 were caused by *C. jejuni* (Silva et al., 2011). In developed countries in general, *C. jejuni* is the most commonly recognized bacterial pathogen. Its numbers even exceeds the combined total number for diarrheal illnesses caused by *Salmonella* , *Shigella* species and *Escherichia coli* (Blaser, 1990). The outcome of infection with *C. jejuni* in developing countries is generally different than developed countries. The infection is endemic especially among children younger than 2 years old and asymptomatic infections commonly occur in older children and adults (Allos, 2001). In studies carried out in Arabian Gulf region, *C. jejuni* accounted for 1.6 to 28% of the diarrhea illness cases in the region (Ismaeel et al., 2002; Senok and Botta, 2009). In Qatar, a recent study focused on the etiology of severe acute gastroenteritis among patients visiting hospitals, *Campylobacter* was isolated from 1.04% of the stool samples tested (Al-Thani et al., 2013).

Despite its clinical importance, many hospital laboratories do not culture routinely for *C. jejuni* and therefore many infections are unreported (Potter et al., 2003). This may be due to the cost and the complex nature of the test compared to the other gastroenteritis causing organisms. Considering the fact that most of the cases are unreported, around 0.5% of the general population may have campylobacteriosis unknowingly (Snelling et al., 2005). *Campylobacter* infections have no clear racial predilection and they are isolated more frequently from males than females (Pasternak et al., 1984). However, studies have shown that

there is a high incidence of *C. jejuni* infection in children who are younger than 1 year and in people aged between 15 to 29 years (Martin et al., 1989). In the United States, the highest incidence of *Campylobacter* infection in 2010 was in children younger than 5 years (Nyati and Nyati, 2013) and in developing countries, the infection is also most common in the first 5 years of life (Allos, 2001).

Source and Transmission of Infection

C. jejuni can live in the intestine of livestock as a part of the normal flora. These animals include poultry, cattle, sheep, and swine (Penner, 1988). *C. jejuni* can directly infect humans by ingestion of contaminated food products of animal origin and consumption of untreated and infected water. However, the consumption and handling of contaminated chickens remains the single most important route of *Campylobacter* infection. Studies from the United States (Harris et al., 1986 , Europe (Jore et al., 2010), and Australia (Stafford et al., 2008) have estimated that 50 to 70% of the human infections could be due to the consumption of chicken. It has been estimated that one drop of the chicken juice contains 500 infectious organisms which might be sufficient to cause infection as *C. jejuni* has a low infective dose (Black et al., 1988; Harris et al., 1986). *C. jejuni* bacteria can't survive under very high temperatures, so good cooking of chicken is emphasized as measure of food safety (Blaser et al., 1983).

Symptoms and Complications

Soon after exposure to *C. jejuni*, the lower intestinal tract (including ileum, jejunum and colon) might become colonized without symptoms. However, in symptomatic cases the disease is self-limiting and is presented with 1 to 3 days of fever, vomiting, headaches followed by 3 to 7 days of watery or bloody diarrhea, bowel movements, and abdominal pain (Dasti et al., 2010). Most of these signs are common to other acute gastrointestinal infections cause by other enteric pathogens and therefore are clinically indistinguishable (Al-Thani et al., 2013).

There is a great variety in the severity of the disease which ranges from mild symptoms to sever dehydration that may require hospitalization. Some of the complications associated with campylobacteriosis include reactive arthritis, bacteremia, endocarditis, cholecystitis, urinary tract infection, pancreatitis and Guillain-Barre syndrome (GBS) (Allos, 2001). GBS is a serious, fatal autoimmune disorder which occurs when the body's immune system mistakenly attacks part of the nervous system (Godschalk et al., 2004). Lipooligosaccharide structure of *C. jejuni* resembles human neuronal ganglioside and that may lead to this autoimmune disorder that approximately affect 1 in every 1,000 cases of campylobacterosis (Hughes, 2004).

Without complications, deaths are rare and most of death cases are in infants, elderly and patients with underlying illnesses (Allos, 2001).

Diagnosis

C. jejuni is a slowly growing fastidious Gram negative organism that requires specific incubation conditions. Methods used for identification of *C. jejuni* involve the use of selective culture media with the combination of biochemical tests like hippurate hydrolysis. Selective media are very useful for the initial isolation of *Campylobacter*, however biochemical methods of identification are necessary for confirmation (Englen and Fedorka-Cray, 2002; Wang et al., 2002). Selective media include blood-free media (such as charcoal cefoperazone deoxycholate agar) or blood containing media like Skirrow media (Corry et al., 1995). Isolation of the organism is done by culturing it in microaerophilic conditions and in incubation temperature of 42°C on a selective media. To enhance the recovery of *C. jejuni* from a stool sample, the use of enriched broth cultures has been recommended (Cox et al., 2001).

Several investigators have introduced the application of multiplex polymerase chain reaction (PCR) for the identification of *C. jejuni* infection. Their approach focuses on using specific virulence related genes for identifications. However, these protocols still required the conventional methods for isolating the organism using selective media and DNA extraction (Al Amri et al., 2007). A recent study has come up with a novel approach in which they used multiplex PCR by combining genus specific virulence genes such as *cadF* with species specific genes such as *hipO* to detect *C. jejuni* directly from stool specimens (Al Amri et al., 2007). This procedure has a turnaround time of 6 hours (from DNA extraction to gel electrophoresis) whereas the conventional methods require two

to five days (LaGier et al., 2004). Therefore, the use of molecular techniques can improve the clinical management and epidemiological tracking of *Campylobacter* infections.

Pathogenesis and Virulence Factors

The exact steps of *C. jejuni* infection that cause enteritis in humans are still unknown. However, the requirements for *C. jejuni* virulence have been observed and they include motility (Guerry, 2007), resistance to antimicrobials (Moore et al., 2006b), adherence to host cells and invasion (Konkel et al., 2001), alteration of the host cell signaling pathways and induction of host cell death (Hickey et al., 2000), and finally iron acquisition (van Vliet et al., 2002).

In the human host, cellular injury happens as a result of cell invasion which leads to reduction in the absorptive capacity of the intestine, whereas CDT plays a major role in the release of interlukin-8 which has an important role in the host mucosal inflammatory response caused by *C. jejuni* (Hickey et al., 2000). CDT consists of three subunits: the catalytic part CdtB, and the binding proteins CdtA and CdtC, which deliver CdtB into the nucleus of target cells (Hickey et al., 2000). Genotoxic effects on the host DNA is induced by the translocation of CdtB to the nucleus which in turn triggers the DNA repair cascade that lead to cell cycle arrest and eventual cell death (Jain et al., 2008; Konkel et al., 2001)

C. jejuni synthesizes a set of proteins known as *Campylobacter* invasion antigens (Cia proteins) that contribute to the invasion of epithelial cells (Konkel et al., 1999; Konkel et al., 2004). The mechanism of secretion of these antigens

(mainly CiaB) and their exact role in invasion has been linked to the model of type III secretion systems, in which effectors are injected directly into host cells (Guerry, 2007). However, *C. jejuni* does not encode a type III secretion system, (Rivera-Amill et al., 2001) but utilizes a flagellar export apparatus to secrete the Cia set of proteins CiaA–H (Konkel et al., 2004). FlaC another protein required for invasion is also secreted from the flagellar export apparatus of *C. jejuni* (Konkel et al., 2004).

For adherence to intestinal epithelial cells, *C. jejuni* possesses a 37 kDa adhesion protein, called fibronectin-binding outer membrane protein (CadF) that binds to the cell matrix protein fibronectin and supports the adherence of *C. jejuni* to the intestinal epithelial cells (Konkel et al., 1997).

The diversity of the clinical outcome of infection by different isolates of *C. jejuni* could be related to the difference in their genetic composition (Carvalho et al., 2001). In 2006, molecular characterization of *C. jejuni* isolates from Bahrain showed a significant relationship between the presence of combinations of virulence genes and the severity of symptoms. *C. jejuni* strains that were positive for more than one of the virulence genes such as *cia, iam* and *cdtB* were isolated from patients with the most severe infections (Al-Mahmeed et al., 2006). However, the isolates which were negative for *cdtB* and *iam* still caused clinical symptoms among children less than 3 years old. This could be due to other unidentified virulence genes that have an effect on the clinical outcome (Al-Mahmeed et al., 2006).

Response to Stress

Due to its complex nutritional requirements, *C. jejuni* has limited capacity of growth in the environment. Unlike other enteric pathogens, *C. jejuni* lacks many adaptive responses which make it sensitive to different environmental stress (Hofreuter et al., 2008). The genome analysis of *Campylobacter jejuni* showed that it lacks the global regulator RpoS which is essential for the survival of many Gram negative bacteria during exposure to environmental stress (Parkhill et al., 2000). However, it has been shown that *Campylobacter* can somehow adapt to both acidic and aerobic conditions (Chaveerach et al., 2003). Exposure to acidic conditions may trigger a conversion of *C. jejuni* into a viable but non-culturable (VBNC) form (Murphy et al., 2003).

During the transmission of *C. jejuni* from its primary reservoirs to the consumer, the bacteria become exposed to environmental changes like oxygen availability and temperature fluctuations. The bacterium has to adapt to these changes in order to survive (Cohn et al., 2007). The consequences that follow these changes trigger the production of chaperones and proteases that play vital roles in the survival of the bacteria by refolding or degrading the proteins that have been damaged by stress. ClpP is an ATP dependent protease that degrades the stress damaged proteins in an energy dependent manner (Hlavacek and Vachova, 2002).

Another gene that is essential for *C. jejuni* is the *htrB* gene which is located in the lipo-oligosaccharide synthesis gene cluster and encodes an

acyltransferase that contributes to lipid A synthesis (Gilbert et al., 2002; Parkhill et al., 2000). This gene plays a major role in regulating how the organism responds to various environmental changes (Phongsisay et al., 2007).

Resistance to Antimicrobials

C. jejuni infection is self-limiting and does not require treatment in most cases. Maintaining hydration and electrolytes balance are the cornerstone treatment for this infection (Kirkpatrick and Tribble, 2011). However, there are some cases that require the use of antimicrobial agents. These cases include high fever, prolonged illness that last more than a week and bloody diarrhea (Kirkpatrick and Tribble, 2011).

The antimicrobial agent of choice for *Campylobacter* infections is erythromycin followed by ciprofloxacin(Nachamkin et al., 2002). Despite being used for decades, now *C. jejuni* showed low rates of resistance to erythromycin (Nachamkin et al., 2002). The advantages of this antimicrobial agent include its low cost, easiness and safety of administration in addition to having a narrow spectrum of activity. Also unlike fluoroquinolones and tetracyclins, it is safe to administer erythromycin to children and pregnant women and is less likely to cause inhibition of normal flora (Saenz et al., 2000).The excessive use and the administration of fluoroquinolones to animals' feed in countries like Holland, Finland, and Spain have led to an increase to antimicrobial resistance among *C. jejuni* strains (Endtz et al., 1991). Also in the UK, the approval of using

fluoroquinolones in domestic pets resulted in an increase in human resistance to the drug (Sam et al., 1999).

Chapter 3 - Materials and Methods

Ethical Approval

Human clinical samples were used in this study. Ethical approval was obtained from the Medical Research Center at Hamad Medical Corporation, protocol number: 13334/13.

Sample Size

The sample size of this study was 174 *C. jejuni* clinical isolates. These isolates were obtained from stool specimens of outpatients showing symptoms of severe diarrhea presented to Hamad General Hospital. *Campylobacter* culture was done at the Microbiology Laboratory of HMC only for specimens of patients who had at least three episodes of sever diarrhea in one day. After confirmation of *C. jejuni* infection by using selective media and biochemical testing, positive isolates were cryopreserved on beads and stored at -80 °C in 1 % (w/v) proteose peptone water containing 10 % (v/v) glycerol until required. Isolates were collected in the period between 2005 and 2011.

Bacteria Culture

The revival of the archived samples was carried out using standard microbiological methods used in HMC. *C. jejuni* isolates were cultured on selective Colombia agar plates (Sigma) and they were grown under microaerophilic conditions using Campy GasPak (BD) at 42°C for 48 hours in an anaerobic jar (BD). Growing colonies were checked for morphology, color, and

size. Only colonies grey or colorless, pinpoint flat or mucoid to convex were used for DNA extraction

DNA Extraction

DNA was extracted from *C. jejuni* isolates following standard molecular biological techniques. Qiagen's mericon DNA Bacteria Kit was used according to the manufacturer's recommended procedures. Positive colonies were suspended with 1 ml of water and collected in a 2 ml microcentrifuge tube then centrifuged at 13,000 x g for 5 min. The supernatant was discarded using a pipet, taking care to not disrupt the pellet. After that, 200 µl of fast lysis buffer was added to the pellet and the pellet was resuspended by vigorous vortexing. Microcentrifuge tubes were placed into a thermal shaker with 800 rpm and a temperature set to 100°C for 10 minutes. The samples were later allowed to cool down for 2 minutes to room temperature (15 - 25°C).Tubes were centrifuged at 13,000 x *g* for 5 minutes. A total of 100 µl of the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and stored at -80°C until used for PCR reaction.

Real-time PCR

RT-PCR was performed using the DNA extracted from the clinical isolates. The primers (Table 1) were designed using primer design software based on available sequences in GenBank. The primers were tested on *C. jejuni* ATCC 33560 strain to confirm their efficiency. For each reaction the following conditions were used: A total of 7 µl of Sybrselect (LifeTechnologies) master mixture was used in each reaction along with 0.3 µl primer mixture, 2.5µl specimen DNA, and

10.2µl molecular grade water. RT-PCR cycles consisted of holding for 2 minutes at 95°C, followed by 40 cycles of 95°C for 15s, 55°C for 35 sec, and 72°C for 1 minute. The cycling conditions for the RT-PCR run is shown in (Table 2)

Table 1: Primers for virulence genes used in this study.

Primers		Sequence (5′-3′)
cadF	Forward	TAT GGT GTA GAA AAA AGT CGC ATC A
	Reverse	ATC CGC TCT ACC TTC TTT AGT GTC A
cdtB	Forward	AAT GCA AGC TGA AGA AGT GAT TGT
	Reverse	AGC ATC ATT TCC ATT GCG AAT
ciaB	Forward	CAA CTT TAT ATT TGC ACT CCG ATG
	Reverse	GGA ACG ACT TGA GCT GAG AAT AAA C
clpP	Forward	TGG GAG CAT TTT TGC TTA GTT G
	Reverse	CTC CAC CTA AAG GTT GAT GAA TCA T
htrB	Forward	CGC ACC CAA TTT GAC ATA GAA C
	Reverse	TTT TTA GAG CGC TTA GCA TTT GTC T

Table 2: RT-PCR cycling conditions.

Step	Time	Temp
Initial heat activation	5 min	95
Denaturing	30 seconds	94
Annealing	40 seconds	60
Extension	60 seconds	72
Number of cycles	42	

Antimicrobial Susceptibility Testing

Minimum inhibitory concentration MIC of antimicrobial agents was determined using the Epsilometer test (bioMerieux). This test uses a rectangular strip that is impregnated with the targeted antimicrobial agents including chloramphenicol, ciprofloxacin, erythromycin and gentamicin. Isolate to be tested was suspended in Brain-Heart Infusion broth and vortexed briefly. The bacteria was spread and grown on an agar plate, and the strip was laid on top. The antimicrobial agents diffused into the agar, making an exponential gradient of the targeted agents. On the strip there is an exponential scale. After incubation for 24 hours, an elliptical zone of inhibition was seen on the plate and the point at which the ellipse meets the strip gave the reading for MIC.

Demographic Data

Information about age, gender and nationality were collected from the electronic medical records of HMC (Medicom). Each subject was assigned a unique identifying code number and patients' identities were not disclosed in the

collected data. All papers and files concerning the study were kept locked in a file cabinets and they were only accessible by the investigators. All data were stored on a password-protected computer that is accessible only by the research team. No identifying information appeared in any of the publications or reports and the data will be destroyed upon the completion of this study.

Statistical Analysis and Validation

Variables studied in this project included: Age, which was continuous and obtained from the Electronic Medical Records of HMC, gender, nationalities which were divided into 4 groups (1. Arabian Peninsula, 2. East and North Africans, 3. Asians and 4. Others), virulence genes categorized as (presence / absence) and finally the results of antimicrobial susceptibility testing which were categorized as (sensitive / resistant).

The sample size of this project was 174 clinical isolates of *C. jejuni*. Only samples which were positive after the reviving and sub-culturing were included in this study and a number of 14 samples of negative culture were excluded. Data were entered into an excel sheet and significance of associations between the variables was tested through bi-variable analysis using Pearson Chi-square test. Fisher exact test was employed for small size samples especially in the sub groups of age and nationalities. *P* values less than 0.05 were considered significant. Analysis was performed using IBM SPSS software, version 22.0.

Chapter 4 - Results

Demographic Characteristics of the Study Population

Age Distribution

The distribution of patients according to age was as the following: Group 1 (5 ≥) contained the majority of patients as it accounted for 75 % of them, Group 2 (6 – 20) 12.6%, Group 3 (21 – 60) 8.6%, and finally Group 4 (61≤) which contained the least number of patients (3.4%)(Table 3).

Gender Distribution

Out of 174 isolates, the prevalence of *C. jejuni* in males was higher as 60.3% of the isolates came from males while 39.7% were obtained from females (Table 3).

Patient Nationality Distribution

Subjects were classified according to their nationalities into 4 groups. Group 1 included people from the Arabian Peninsula. Group 2 had subjects who are originally from North and East Africa. Group 3 were Asians and the Group 4 contained people living in areas other than the above mentioned. In the present study, the majority of patients were from the Arabian Peninsula (57%) followed by Asians (28%), while East and North Africans comprised 11.5% and people from other origins accounted for only 3.5% (Table 3).

Table 3: Demographic characteristics of the study population including age groups, gender and nationalities.

	No. of patients (%)
Age groups	
5 ≥	131 (75)
6-20	22 (12.6)
21-60	15 (8.6)
61≤	6 (3.4)
Median age: 2 (0.08 – 75) years.	
Gender	
Male	105 (60.3)
Female	69 (39.7)
Nationalities	
Arabian Peninsula	99 (57)
East and North Africans	20 (11.5)
Asians	49 (28)
Others	6 (3.5)

Antimicrobial Susceptibility Testing

All clinical isolates were tested for their antimicrobial susceptibility to the antimicrobial agents commonly used at HMC which contained: chloramphenicol, ciprofloxacin, erythromycin and gentamicin. Apart from ciprofloxacin, all isolates were highly sensitive to the antimicrobials with percentages above 90%. All isolates were susceptible to gentamicin (Table 4).

Table 4: Antimicrobial susceptibility testing results for *C. jejuni* isolates.

	Chloramphenicol n (%)	Ciprofloxacin n (%)	Erythromycin n (%)	Gentamicin n (%)
Sensitive	173 (99.4)	64 (36.7)	159 (91.4)	174 (100)
Resistant	1 (0.6)	110 (63.3)	15 (8.6)	0 (0)

Prevalence of Virulence Genes

Presence of the virulence-related genes *cadF*, *cdtB* and *ciaB* in the isolates was determined. *cdtB* was the most prevalent (87.36%) followed by *ciaB* (71.8%) and finally *cadF* (59.8%)(Table 5). Stress response genes, *clpP* and *htrB*, were also examined and both were detected in the *C. jejuni* isolates with percentages of 67.8% and 83.3%, respectively (Table 5).

Table 5: Prevalence of virulence genes in *C. jejuni* isolates. Of the five genes, *cdtB* was the most prevalent while *cadF* was the least.

Virulence Genes	No. of isolates positive for each gene by RT-PCR (%)
ciaB	125 (71.8)
cadF	104 (59.8)
cdtB	152 (87.36)
htrB	118 (67.8)
clpP	145 (83.3)

Further analysis was done to study the presence of more than one gene of the genes associated with the virulence mechanism at the same *C. jejuni* strain.

Almost 65% of the isolates harbored both *ciaB* and *cdtB*, while 51.7% had the genes *cadF* and *ciaB* and 55.1% carried *cadF* and *cdtB*. Only 33.9% of the isolates expressed all the virulence genes tested in this study at the same time (Table 6).

Table 6: Prevalence of virulence factors genes combination in same *C. jejuni* isolate. The most prevalent gene combination was *ciaB* and *cdtB*, while *ciaB* and *cadF* combination was the least prevalent.

Virulence Genes	No. of isolates positive for genes by RT-PCR (%)
ciaB (+), *CadF* (+), *cdtB* (+)	59 (33.9)
ciaB (+), *CadF* (+)	90 (51.7)
ciaB (+), *cdtB* (+)	113 (64.9)
cadF (+), *cdtB* (+)	96 (55.1)
clpP (+), *htrB* (+)	105 (60.34)

Association of Virulence Gene Presence with Gender, Age and Nationality

The next approach was to examine whether there are any associations between the presences of the target genes and the demographic data collected from the patients including gender, age and nationality. There was a significant association between the gender (male) and the presence of *cadF* and *clpP* genes with a *P* value of 0.022 for both. However there was no significant correlation between the gender and remaining genes. Also there were no clear associations between patients' nationalities and the presence of both virulence genes (Table 7). Furthermore, the presence of the genes studied in this project

was tested for associations with the different age groups. However no significant association was found between presence of target genes and age.

Table 7: Distribution of virulence gene presence by gender, age groups and nationality.

	cadF (+) (104) n (%)	*ciaB* (+) (125) n (%)	*cdtB* (+) (152) n (%)	*clpP* (+) (145) n (%)	*htrB* (+) (118) n (%)
Gender					
Male (105)	70 (66.7)*	80 (76.2)	95 (90.5)	93 (88.6)*	71 (67.6)
Female (69)	34 (49.3)	45 (65.2)	57 (82.6)	52 (75.4)	47 (68.1)
Age groups					
5 ≥ (131)	81 (61.8)	97 (74)	114 (87)	109 (83.2)	89 (67.9)
6-20 (22)	10 (47.6)	10 (47.6)	19 (90.5)	18 (85.7)	13 (61.9)
21-60 (15)	9 (64.3)	12 (85.7)	11 (78.6)	11 (78.6)	9 (64.3)
61≤ (6)	6 (75)[a]	7 (87.5)[a]	8 (100)[a]	7 (87.5)[a]	7 (87.5)[a]
Nationality					
Arabian Peninsula (99)	61 (62.2)	73 (74.5)	87 (88.8)	81 (82.7)	64 (65.3)
East and North Africans (20)	13 (61.9)	13 (61.9)	17 (81)	14 (66.7)	12 (57.1)
Asians (49)	24 (49)	34 (69.4)	42 (85.7)	45 (91.8)	36 (73.5)
Others (6)	6 (100)[a]	5 (83.3)[a]	6 (100)[a]	5 (83.3)[a]	6 (100)[a]

*Using Pearson chi square, *P* value < 0.05 was considered significant.

[a] Fisher exact test was used for small size samples.

Association of Antimicrobial Susceptibility with Gender, Age and Nationality

For antimicrobial sensitivity in relation to patient gender and nationality, no significant associations were recorded (Table 8). Also, antimicrobial

susceptibility results did not have any significant associations with the different age groups included in this study (Table 8).

Table 8: Distribution of antimicrobial sensitivity by gender, age and nationality.

	Chloarmphenicol (173) n (%)	Ciprofloxacin (69) n (%)	Erythromycin (159) n (%)	Gentamicin [a] (174) n (%)
Gender				
Male (105)	104 (99)	41 (39)	97 (92.4)	105 (100)
Female (69)	69 (100)	23 (33.3)	62 (89.9)	69 (100)
Age groups				
5 ≥ (131)	130 (99.2)	51 (38.9)	120 (91.6)	131 (100)
6-20 (22)	22 (100)	7 (31.8)	20 (90.9)	22 (100)
21-60 (15)	15 (100)	5 (33.3)	13 (86.7)	15 (100)
61≤ (6)	6 (100)[b]	1 (16.6)[b]	6 (100)[b]	6 (100)[b]
Nationality				
Arabian Peninsula (125)	98 (100)	39 (39.8)	89 (90.8)	98 (100)
East and North Africans (20)	21 (100)	8 (38.1)	18 (85.7)	21 (100)
Asians (49)	48 (98)	15 (30.6)	46 (93.9)	49 (100)
Others (6)	6 (100)[b]	2 (33.3)[b]	6 (100)[b]	6 (100)[b]

[a]. No statistics are computed because gentamicin is constant (all isolates were sensitive).

[b]. Fisher exact test was used for small size samples.

*Using Pearson chi square, *P* values < 0.05 was considered significant.

Association of Antimicrobial Susceptibility with Presence of Target Genes

We tested whether the difference in prevalence of the different genes studied in this project has a role in the antimicrobial susceptibility patterns. No significant correlations were found between the results of antimicrobial susceptibility test and the prevalence of the virulence genes. However, it seems

that there is a weak association of ciprofloxacin resistance with the presence of the gene *htrB* (*P* value 0.069) (Table 9).

Table 9: Virulence genes presence by antimicrobial sensitivity.

Antimicrobial Agents	*cadF* (+) n (%)	*ciaB* (+) n (%)	*cdtB* (+) n (%)	*clpP* (+) n (%)	*htrB* (+) n (%)
Chloramphenicol	103 (59.5)	124 (71.7)	151 (87.3)	144 (83.2)	117 (67.6)
Ciprofloxacin	35 (54.7)	43 (67.2)	57 (89.1)	55 (85.9)	80 (72.7)
Erythromycin	97 (61)	114 (71.7)	140 (88.1)	135 (84.9)	110 (69.2)
Gentamicin	104 (59.8)[a]	125 (71.8)[a]	152 (87.4)[a]	145 (83.3)[a]	118 (67.8)[a]

[a]. The correlation was not tested in gentamicin because all of the isolates were sensitive to the agent.

*Using Pearson chi square, *P* value < 0.05 was considered significant.

Chapter 5 - Discussion

Campylobacter jejuni remains a major cause of gastroenteritis worldwide. The emergence of these bacteria as a main source for human disease and their ability to show antimicrobial resistance patterns have contributed in making *C. jejuni* infections a serious public health concern (Dasti et al., 2010).

Most of the studies that have focused on the prevalence of *Campylobacter jejuni* virulence associated genes were done using isolates from Europe, United States and Latin America (Gonzalez-Hein et al., 2013). As far as we know, no previous research was done using isolates from Qatar. In this study, a total of 174 *C. jejuni* isolates were tested for the presence of several virulence and stress-response factors in addition to antimicrobial resistance for the agents commonly used at HMC. These samples were archived by the Microbiology Laboratory at HMC during the period from 2005 to 2011.

Our findings showed that subjects who were five years old or younger were the most vulnerable population for *C. jejuni* infection as 75% of the patients in this study belonged to this age group. This finding is consistent with previous studies done in the United States (Nyati and Nyati, 2013) and in several developing countries (Allos, 2001; Kotloff et al., 2013). In our findings, the slight predominance of male subjects (60.3% to males and 39.7% to females) was also consistent with gender-related susceptibility to *C. jejuni* infection in previous studies (Allos, 2001). The reason of this specific age and gender distribution remains unknown.

In this study, 57% of *C. jejuni* isolates belonged to subjects from Arabian Peninsula followed by Asians with 28%, then East and North Africans (11.5%) and finally subjects from other nationalities (3.5%). Although the numbers of expatriates in Qatar exceeds the number of nationals, our results showed that the majority of subjects were from Arabian Peninsula. This predominance could be due to access to health services rather than any true epidemiological differences.

The pathogenicity of *C. jejuni* is complex and multi-factorial as several genes are involved in the progression of the disease (Ketley, 1997). In this study, five virulence associated genes have been investigated in isolates collected from the Microbiology Laboratory at HMC. The genes investigated in this study were: *cadF*, *cdtB*, *ciaB*, *clpP* and *htrB*. They are important for adhesion, toxin production, invasion and stress response (van Vliet and Ketley, 2001). The genes of interest were detected in all *C. jejuni* isolates from HMC with different frequencies. *cdtB* gene was by far the most prevalent as it was present in 87.3% of the isolates. *cdtB* encodes the catalytic part of CDT, which is responsible for the genotoxic effect on the DNA of the cell (Hickey et al., 2000; Jain et al., 2008; Konkel et al., 2001). The CdtB subunit along with the two binding subunits, CdtA and CdtC, forms the CDT protein which also induces an inflammatory response at the mucosal surfaces (Hickey et al., 2000). *ciaB* was also present in 71.8% of *C. jejuni* isolates. Out of the 9 *Campylobacter* invasion antigens, (CiaA-H), CiaB is probably the most important protein as its deletion abolishes the ability of *C. jejuni* to invade cells (Konkel et al., 1999; Konkel et al., 2004). *C. jejuni* does not

encode a type III secretion system, (Rivera-Amill et al., 2001) but utilizes a flagellar export apparatus to secrete the Cia set of proteins and other proteins and inject them directly into host cells (Guerry, 2007; Konkel et al., 2004). The high prevalence of *cdtB* and *ciaB* genes is consistent with other studies where these two genes are generally widespread among isolates from humans with clinical symptoms (Fearnley et al., 2008). The prevalence of *cadF* gene, however, was only 59.8% in the isolates. This finding was inconsistent with other studies where *cadF* was found to approach a prevalence of 100% in *C. jejuni* clinical isolates (Fearnley et al., 2008). The two genes responsible for stress response *clpP* and *htrB* were also present with frequencies of 83.3% and 67.2% respectively. In *Escherichia coli*, mutation of *htrB*, which encodes a lipid A acyltransferase, causes a variety of effects that include a change in the morphology of rods (Karow et al., 1991), growth inhibition at higher temperatures (Karow et al., 1991), and several fold-increase in bile (Karow and Georgopoulos, 1992). The same effects are observed in *Salmonella* in addition to hyperflagellation and loss of virulence (Jones et al., 1997). In *Haemophilus influenzae*, knockout of *htrB* did not affect the morphology of the bacterium but rather its ability to colonize and survive intracellularly (Swords et al., 2002). The deletion of *htrB* in *C. jejuni* reduces its capacity to grow well at the higher temperature of 42°C, which is the body temperature of chickens (Phongsisay et al., 2007). The deletion of this gene also affected *C. jejuni*'s resistance to acid and osmotic pressure (Phongsisay et al., 2007). This reflects the importance of these genes for the growth of *C. jejuni* in gastrointestinal tract of humans.

Infections caused by *C. jejuni* are typically self-resolving and they do not require treatment. However in severe cases, the administration of antimicrobial agents may be necessary for patients with prolonged and systemic infections (Kirkpatrick and Tribble, 2011). Macrolides like erythromycin and fluoroquinolones like ciprofloxacin are currently considered the antimicrobial agents of choice (Slutsker et al., 1997). However, recent studies showed varying patterns of *Campylobacter* resistance to antibiotics in different regions. Furthermore, there is a concern about the widespread use of antimicrobial agents such as erythromycin, ciprofloxacin and tetracycline in animal feed. This has helped the generation of several *C. jejuni* strains that are resistant to antibiotics (Moore et al., 2006a). In HMC, the list of common antimicrobial agents used to control the infection include: erythromycin, ciprofloxacin, chloramphenicol and gentamicin. The antimicrobial susceptibility patterns were tested in the clinical isolates. Isolates were highly sensitive to three out of the four antibiotics tested in this study: chloramphenicol, gentamicin and erythromycin. In the case of gentamicin, there was no resistance as all the isolates were sensitive to this agent. This was consistent with studies done in New Zealand and the United States where isolates were sensitive to this antimicrobial (Moore et al., 2006a). On the other hand, 64.7% of the isolates were resistant to ciprofloxacin. Ciprofloxacin resistance was also determined in studies carried out in South Africa and Lebanon (Moore et al., 2006a). A strong association has been observed between the licensing of fluoroquinolones for use in animal feed in Europe and the increase of antimicrobial resistance against this

particular agent (Endtz et al., 1991). The same link was also made in the United Kingdom when this family of drugs was used in pets as human isolates started showing resistance (Sam et al., 1999). Based on our findings, imply the usefulness of erythromycin as the drug of choice in HMC.

One of the main goals of the study was to test associations between the prevalence of virulence genes and demographic data collected from the patients. A significant association between gender (male) and the presence of the genes *cadF* and *clpP* (*P* value: 0.022) was seen. However the same was not found in the remaining genes. Also in our study, the antimicrobial susceptibility patterns had no clear associations with demographic data (age, gender and ethnicity). The analysis was carried out only for ciprofloxacin as the number of isolates resistant to other agents was not sufficient to perform a meaningful statistical analysis. The association between the prevalence of the different genes and whether or not they have an influence on the antimicrobial susceptibility patterns revealed no significant association between antimicrobial sensitivity and the prevalence of virulence genes. A limitation to the study was the small sample size of the sub-groups of age and nationality. This has made it difficult to come up with conclusive results for the tested associations.

When it comes to the pathogenesis of *C. jejuni* infection, little is understood. The prevalence of *C. jejuni* virulence genes and their relationship with the severity of symptoms in humans should be further investigated. As HMC's electronic medical records do not include symptoms of their data and

paper files information were not consistent, an analysis for the association of the symptoms with the prevalence of virulence factors was not carried out.

Chapter 6 - Conclusion

Using relatively significant number of *C. jejuni* isolates, we have shown that the main virulence and stress response genes were present in isolates collected from HMC hospital. The fact that these isolates showed antimicrobial resistance only towards ciprofloxacin but were highly sensitive to chloarmphenicol, gentamicin and erythromycin suggests that the use of this agent in animals is probably contributing to the spread of its resistance. Indeed, laws in Qatar allow the use of enrofloxacin, a related fluoroquinolone, in the poultry industry. Since *C. jejuni* remains a major source and cause of enteritis in Qatar and around the world, further investigation of the molecular characteristics of these bacteria, and how they induce different clinical manifestations and responses to infection is needed.

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Appendix - Ethical Approval

Medical Research Center

Ref No: MRC[illegible] /2014
Date: 7 January 2014

Dr. Sanjay Deiphode
Consultant
Lab Medicine and Pathology

Dear Dr. Diophode,

Research Protocol #13214/13, "Molecular analysis of Campylobacter spp virulence genes from clinical isolates in Qatar"

The above titled Research Proposal submitted to the Medical Research Center has been reviewed and classified as 'Exempt' under SCH guidelines for exempt research and approval is granted from 7 January 2014.

This research study should be conducted in full accordance with all the applicable sections of the rules and regulations for research at HMC and you should notify the Medical Research Center immediately of any proposed protocol changes that may affect the 'exempt' status of your research proposal. It is the Principal Investigator's responsibility to obtain review and continued approval of the proposal if there is any modification to the approved protocol.

Documents reviewed by the Research Center:

- Research Proposal
- Consent form: Waiver of Informed Consent
- Data Collection Form

A study progress report should be submitted bi-annually and a final report upon study's completion.

We wish you all success and await the results in due course.

Yours sincerely,

Dr. Ibrahim A. Janahi,
Executive Director of Research

Cc:

1. Dr. Marwan Abdmad, Assistant Prof., Qatar University
2. Dr. Haitham Shaheen, Post. Doctoral Fellow, Qatar University
3. Mr Agha, Graduate Student, Qatar University
4. Dr. Anand Jeremiah, Specialist, Dept. of Lab Medicine and Pathology
5. Dr. Eltaib Saeed, Consultant, Dept. of Lab Medicine and Pathology
6. Chairman, Dept. of Lab Medicine and Pathology
7. Medical Director, HGH

Tel: (+974) 4439 2460
Fax: (+974) 44392462
research@hmc.org.qa

P.O.Box 3050
Doha, Qatar
www.hmc.org.qa